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Relation	

ORIGINAL INVESTICATION

The Role of Glucocorticoid Receptors in the Induction and Prevention of Hippocampal Abnormalities in an Animal Model of Posttraumatic Stress Disorder

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Conflict of interest

The authors declare that they have no conflict of interest.

Abstract

Rationale

Since the precise mechanisms of posttraumatic stress disorder (PTSD) remain unknown, effective treatment interventions have not yet been established. Numerous clinical studies have led to the hypothesis that elevated glucocorticoid levels in response to extreme stress might trigger a pathophysiological cascade which consequently leads to functional and morphological changes in the hippocampus.

Objectives

To elucidate the pathophysiology of PTSD, we examined the alteration of hippocampal gene expression through the glucocorticoid receptor (GR) in the single prolonged stress (SPS) paradigm, a rat model of PTSD.

Methods

We measured nuclear GRs by western blot, and the binding of GR to the promoter of Bcl-2 and Bax genes by chromatin immunoprecipitation-qPCR as well as the expression of these 2 genes by RT-PCR in the hippocampus of SPS rats. In addition, we examined the preventive effects of a GR antagonist on

SPS-induced molecular, morphological, and behavioral alterations (hippocampal gene expression of Bcl-2 and Bax, hippocampal apoptosis using TUNEL staining, impaired fear memory extinction [FME] using the contextual fear conditioning paradigm).

Results

Exposure to SPS increased nuclear GR expression and GR binding to Bcl-2 gene, and decreased Bcl-2 mRNA expression. Administration of GR antagonist immediately after SPS prevented activation of the glucocorticoid cascade, hippocampal apoptosis, and impairment FME in SPS rats.

Conclusion

The activation of GRs in response to severe stress may trigger the pathophysiological cascade leading to impaired FME and hippocampal apoptosis. In contrast, administration of GR antagonist could be useful for preventing the development of PTSD.

Key words: animal model, posttraumatic stress disorder (PTSD), glucocorticoid receptor (GR), GR antagonist

Introduction

Posttraumatic stress disorder (PTSD), the most widely prevalent stress-related mental disorder, is a tenacious, debilitating, and frequently intractable condition (Committee on the Assessment of Ongoing Efforts in the Treatment of Posttraumatic Stress et al. 2014). Although the precise mechanisms of PTSD are still unknown, dysfunction of the hypothalamo-pituitary-adrenal (HPA) axis is reported to be closely involved in the pathophysiology of PTSD. For example, Yehuda and colleagues demonstrated enhanced negative feedback of the HPA axis in response to dexamethasone in patients with PTSD (Daskalakis et al. 2016; Szeszko et al. 2018; Yehuda et al. 2015), suggesting that HPA axis dysfunction may be due to increased levels of glucocorticoid receptor (GR) in the hippocampus. In addition, it has been reported that the measurement of GR mRNA in lymphocytes prior to traumatic stress exposure predicts incidence of PTSD (van Zuiden et al. 2012), and that lymphocyte GR levels and cytosine methylation levels at the promoter of the GR gene in the blood are increased in patients with PTSD (Yehuda et al., 2015). Similarly, to elucidate the mechanism responsible for the enhanced negative feedback of the HPA axis, Liberzon and coworkers showed that the transient down-regulation of GR mRNA levels (24 h after stress) and subsequent up-regulation of GR mRNA levels (7 days after stress) in the rat hippocampus were associated with enhanced negative feedback in the single prolonged stress (SPS) paradigm, an animal model of PTSD (Liberzon et al. 1999). In addition, it is well known that the activation of GRs alters gene transcription through the binding of glucocorticoid and GR complex to the glucocorticoid-response

element (GRE) at the promoter of various genes (Mifsud and Reul 2016; Necela and Cidlowski 2004; Oakley and Cidlowski 2013). Based on these findings, it is conceivable that elevated cortisol/corticosterone levels due to exposure to severe stress may be crucial to the development of the enhanced negative feedback of the HPA axis. In fact, according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders, Fifth edition (DSM-5), severe stressors must be a predisposing factor to the development of PTSD, and PTSD should be diagnosed more than 1 month after trauma. These criteria indicate that the activation of GRs and subsequent molecular changes are closely involved in the development of PTSD. Thus, to better understand the pathophysiology of PTSD, it is important to clarify the molecular changes that occur in the hippocampus subsequent to the elevation of glucocorticoids in response to stress.

In line with dysfunction of the HPA axis, numerous clinical studies of PTSD have demonstrated functional and morphological abnormalities in the hippocampus after exposure to life-threating trauma (Szeszko et al. 2018), such as reduced hippocampal volume (Nelson and Tumpap 2017; O'Doherty et al. 2015) and impaired fear memory extinction (Milad et al. 2008; Milad et al. 2009; Rothbaum and Davis 2003). Additionally, changes in the expression of genes associated with the apoptotic pathway, such as imbalance of anti-apoptotic Bcl-2 and pro-apoptotic Bax, are reported to be involved in the mechanism of stress related to hippocampal atrophy (Gruver-Yates and Cidlowski 2013; Zhang et al. 2006). Although the precise mechanisms remain unknown, SPS has been shown to induce neuronal apoptosis in the

hippocampus through an imbalance of the Bcl-2/Bax ratio (Li et al. 2010b). These findings suggest that determining how SPS affects Bcl-2 and Bax gene expression through the activation of GR in the rat hippocampus would be helpful for elucidating the pathophysiology of PTSD in humans.

With regard to the treatment of PTSD, combination treatment with selective serotonin reuptake inhibitors (SSRIs) and prolonged exposure therapy have been reported to be effective. However, approximately 30% to 40% of patients with PTSD remain chronically symptomatic and exhibit intrusion symptoms (Boe et al. 2010; Feuer et al. 2005; Friedman et al. 1995; Kessler et al. 1995; Schneier et al. 2012). The effectiveness of psychosocial approaches (psychological debriefing (Litz et al. 2002; Mitchell 1983) and cognitive behavioral therapy (Bryant et al. 1998; Bryant et al. 2005; Bryant et al. 1999; Foa et al. 2006)) and various pharmacological interventions (hydrocortisone (Schelling et al. 2001; Schelling et al. 2004), beta blockers (Pitman et al. 2002; Vaiva et al. 2003), SSRIs (Stoddard et al. 2011), morphine (Bryant et al. 2009; Holbrook et al. 2010), docosahexaenoic acid (Matsuoka et al. 2015), omega-3 polyunsaturated fatty acids (Matsumura et al. 2017), and ketamine (McGhee et al. 2008)) has been investigated for preventing the development of PTSD at an earlier stage after traumatic stress exposure. Hydrocortisone has been regarded to be exceptionally effective in preventing PTSD based on moderate quality evidence from a 2014 Cochrane review (Amos et al. 2014) and the treatment guidelines of the International Society for Traumatic Stress Studies (https://www.istss.org/). Although the protective effects of glucocorticoids against the development of PTSD is still controversial, several preclinical

studies provided the evidence that a modest increase in glucocorticoid levels prior to acute stress exposure prevented the delayed enhancing effect of stress, such as synaptic connectivity in the basolateral amygdala and anxiety-like behavior (Karst et al. 2010; van Zuiden et al. 2012). However, the mechanism by which pharmacological therapies can facilitate extinction learning (Hruska et al. 2014) or enhance synaptic plasticity and connectivity (Zohar et al. 2011) remain hypothetical and the effectiveness of early clinical interventions has not yet been established (Qi et al. 2016). Thus, a better understanding of the underlying pathophysiology of PTSD could facilitate the development of novel strategies for the treatment and the prevention of the disorder.

In the present study, we used the SPS paradigm as an animal model of PTSD. SPS mimics many aspects of the pathophysiological abnormalities associated with PTSD, and also some aspects of the associated behavioral characteristics (Souza et al. 2017; Yamamoto et al. 2009) including impaired fear memory extinction (Keller et al. 2015; Knox et al. 2012). We first examined the effect of SPS on nuclear levels of GRs in rat hippocampus by western blot. Next, we examined the binding of GR to the GRE at the promoters of the Bcl-2 and Bax genes by chromatin immunoprecipitation (ChIP)–qPCR, and we measured the expression of these 2 genes by RT-PCR in the hippocampus of rats after SPS. Lastly, we examined whether administration of a GR antagonist, GU486, immediately after SPS could prevent altered gene expression of Bcl-2 and Bax in the hippocampus, increase hippocampal apoptosis (measured by TUNEL staining), or ameliorate the impaired fear memory extinction in the contextual fear

conditioning paradigm.

Materials and methods

All experimental procedures are shown in Fig. 1.

Animals

Male Sprague–Dawley rats weighing between 300 g and 350 g (Charles River Laboratories Japan, Inc., Yokohama, Japan) were used in this study. The animals were group-housed (3 per cage) and maintained on a 12-h light/dark cycle with food and water freely available. All procedures took place during the light cycle. All animal procedures were conducted in strict accordance with the Hiroshima University School of Medicine's Animal Care Committee Guiding Principles on Animal Experimentations in Research Facilities for Laboratory Animal Science.

Single Prolonged Stress (SPS)

Animals were randomly assigned to either the sham or SPS group. During SPS treatment, sham rats were moved and placed in cages identical to those in which SPS was conducted. SPS was carried out in 3 stages as previously described (Kataoka et al. 2018; Liberzon et al. 1997; Liberzon et al. 1999; Matsumoto et al. 2013): restraint for 2 h, forced swim for 20 min, and ether anesthesia. Briefly, each rat was restrained for 2 h by placing it inside a disposable clear polyethylene cone bag (Asahikasei, Tokyo, Japan) with only the tail protruding. After immobilization, they were individually placed in a clear acrylic cylinder (240 mm diameter, 500 mm height), filled two-thirds from the bottom with water (24°C) and

forced to swim for 20 min. Following 15 min recuperation, they were exposed to diethyl ether until loss of consciousness.

Measurement of the amount of nuclear glucocorticoid receptor (GR) in the hippocampus by western blotting

We first examined whether SPS changed the nuclear levels of GRs in the rat hippocampus 1, 2, and 4 h (10 rats each) after SPS exposure (Fig. 1a). EpiQuik™ Nuclear Extraction Kit II (Epigentek, New York, NY, USA) was used for the preparation of nuclear proteins from hippocampal tissues according to the manufacturer's instructions. The fidelity of the kit to isolate the nuclear extract of the rodent brain had been reported (Siuda et al. 2014; Wood et al. 2015). Total protein concentrations were measured using a BCA kit (Thermo Scientific, USA). Protein (30 μg) was loaded into each lane of 4% to 12 % gradient Bis–Tris SDS NuPAGE gels (Invitrogen, Carlsbad, CA, USA). Proteins were resolved by electrophoresis and blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked at room temperature for 1 h in 1×TBS 1% Casein Blocker (Bio-Rad Laboratories, Tokyo, Japan), and then incubated overnight at 4°C with the primary antibodies diluted in 1×TBS 1% Casein Blocker. Antibodies used included those specific for GR (1:1000, catalogue number #3660; Cell Signaling Technology, Beverly, MA, USA), and TATA binding protein (1:1000, catalogue number #8515; Cell Signaling Technology). After washing with TBST, the membranes were incubated with secondary

antibodies (horseradish peroxidase-conjugated anti-rabbit IgG antibody; GE Healthcare, Little Chalfont, Buckinghamshire, UK) diluted 1:1000 with 1×TBS 1% Casein Blocker for 1 h at room temperature. The membranes were washed again and detected with the chemiluminescene ECL western blotting system (GE Healthcare). The densities of the immunoreactive bands were quantified with Image Lab software (Bio-Rad Laboratories). The GR value was corrected for the amount of TATA binding protein in each sample.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed to measure the extent of GR binding to the GRE at the Bcl-2 and Bax gene promoter sites using a ChIP kit ('Epi Quik', Epigentek) with some modifications (Fig. 1b). Whole hippocampus was minced into 1 mm-sized pieces and immediately crosslinked in 1% formaldehyde for 20 min at room temperature. The crosslinking reaction was stopped by adding glycine. The tissue was washed and centrifuged at 800 rpm for 5 min. The pellet was then collected and homogenized in homogenizing buffer using a Dounce homogenizer (10-20 strokes). The homogenate was again centrifuged at 3000 rpm for 5 min. The supernatant was next lysed in lysis buffer containing protease inhibitor cocktail and the lysates were sonicated 10 times at 30-s intervals using a Bioruptor (Tosho Denki, Tokyo, Japan) at the maximum setting. Sonicated lysates were incubated with 300 U micrococcal nuclease (Fermentas Life Sciences, Burlington, Ontario, Canada), yielding DNA fragments 200 to 1000

bp in length. After the chromatin lysate was extracted and properly fragmented, chromatin lysates were diluted with ChIP dilution buffer. Aliquots (5 μL) of pre-immunoprecipitated diluted supernatant were saved as "input" DNA for later normalization. The chromatin solution was then immunoprecipitated at room temperature for 90 min with 5 μg of antibody directed against GR (catalogue number #12041, Cell Signaling Technology). The specificity of this antibody in ChIP assays has been previously established (Gao et al. 2015). As negative and positive controls, samples were immunoprecipitated with 1 μg nonimmune rabbit IgG and anti-RNA polymerase, respectively. Chromatin was then extracted and eluted from the columns with DNA release buffer containing protease K and elution buffer.

Quantification of GR binding to GRE by real-time PCR

GR binding to the GRE at the Bcl-2 and Bax gene promoter regions was quantified by measuring the amount of GR-immunoprecipitated DNA using quantitative real-time PCR with an ABI Prism 7900 sequence detection system (PE Applied Biosystems). Specific primers and TaqMan MGB hybridization probes were designed and synthesized by Applied Biosystems to amplify the GRE of each gene explored using the EpiTect ChIP qPCR Primers transcription factors search tool from Qiagen (Qiagen EpiTect ChIP qPCR Primers Transcription Factor, SABiosciences.). The primers and probes used are shown in Table 1. The PCR assay for 'input' was performed for normalization at the same time. The PCR assay for unknown samples was performed simultaneously with standard ChIP samples (rat hippocampus) to

construct a standard curve. The relative concentrations of genes in unknown samples were calculated from this standard curve, and the ratios of the relative concentrations of these genes were calculated relative to the concentration of 'input' DNA.

Measurement of Bcl-2 and Bax mRNA levels in the hippocampus by real-time RT-PCR

To determine the effects of SPS on the expression of apoptosis-associated genes in the hippocampus, we next performed RT-PCR to measure alterations in Bcl-2 and Bax mRNA levels (Fig. 1b). Total RNA was extracted from the hippocampus with an RNAqueous Phenol-free Total RNA Isolation kit (Ambion, Austin, TX, USA). Single-stranded cDNA was synthesized using the QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany). Levels of total Bcl-2 and Bax mRNA were determined by quantitative RT-PCR using the ABI Prism 7900 sequence detection system (PE Applied Biosystems). Specific primers and TaqMan probes were designed and synthesized by Applied Biosystems to amplify total cDNA of each gene. The primers and probes used are shown in Table 2. All samples were assayed in triplicate. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) quantification was performed using the TaqMan Rodent GAPDH Control Reagent kit (PE Applied Biosystems) as an internal control for normalization. The PCR assay for unknown samples was performed simultaneously with standard samples (rat hippocampus) to construct a standard curve. The relative concentrations of GAPDH and genes in unknown samples were calculated from this standard curve, and the ratios of their relative concentrations

were calculated relative to the concentration of GAPDH.

TUNEL staining

To detect apoptotic cells in the hippocampus, TdT-mediated dUTP nick end labeling (TUNEL) staining was performed using an In Situ Cell Death Detection kit, POD (Roche Diagnostic GmBH, Mannheim, Germany). One week after SPS, rats were deeply anesthetized with chloral hydrate (250 mg/kg, i.p.) and transcardially perfused with ice-cold PBS followed by 10% buffered formalin (Fig. 1b). Following perfusion, brains were removed and post-fixed in the same fixative for 24 h to 48 h at 4°C, cryoprotected in 30% sucrose-tris buffered saline (TBS) for 48 h to 72 h, and then sectioned at a thickness of 40 μm. The sections were made at approximately 3.8 mm (dorsal hippocampus [dHPC]) and 5.6 mm (ventral hippocampus [vHPC]) posterior to bregma (Paxinos and Watson 1998). The sections were incubated sequentially with paraformaldehyde for 10 min at room temperature, 0.6% TBST for 60 min at room temperature, proteinase K for 30 min at 37°C, 50 μL of TUNEL reaction mixture for 60 min at 37°C, and 50 μL of converter-POD for 30 min at 37°C. Subsequently, sections were stained using a DAB Peroxidase Substrate Kit, ImmPACT (Vector Laboratories, Burlingame, USA), then counterstained with Methyl Green Solution (Fujifilm Wako Pure Chemical Industries, Osaka, Japan). Four microscopic fields per region in CA1, CA3, and the dentate gyrus (DG) were captured using a Keyence BZ-X800 fluorescence microscope (Keyence Corporation, Osaka, Japan). The camera exposure and gain setting were held

constant between sections. The ratio of apoptotic cells was determined as the number of TUNEL-positive cells divided by the total cell number (TUNEL-positive plus methyl green-positive cells). The Hybrid Cell Count algorithm for the Keyence BZ-X800 was used to assess TUNEL-positive and methyl green-positive cells.

Fear conditioning test (FCT)

Contextual fear conditioning, extinction training, and extinction test were performed as previously described (Kataoka et al. 2018) (Fig. 1c). On Day 7, rats were placed in a conditioning chamber (325 mm width, 280 mm height, 500 mm depth), and then were exposed to a 180-s conditioning context without any stimulation. Immediately after that, they received a 4-s, 0.8-mA footshock through a stainless steel grid floor by a shock generator-scrambler (SGS-003: Muromachi, Tokyo, Japan). Two footshocks were delivered with an inter-trial interval of 3 min. Following the footshocks, rats remained in the chamber for an additional 1 min before being returned to their home cages. Extinction training was defined as the repetitive exposure to the contextual cue (the chamber) in the absence of footshock. On Day 8, rats were placed for 10 min without footshock in the same chamber where the footshock was delivered. Extinction recall was tested on Day 9 under conditions identical to extinction training.

Freezing time was monitored and a percentage score was calculated for the proportion of the total observation period spent freezing. Freezing behavior of each rat was recorded on videotape and later

scored blindly by well-trained experimenters.

Drug

We used mifepristone (RU486) (TCI Chemicals, Tokyo, Japan) as a GR antagonist. RU486 antagonizes the function of GRs through 2 mechanisms; competition with corticosterone in the cytoplasm, and competition of the RU486-GR complex with corticosterone-GR complex in the nucleus for DNA binding (Peeters et al. 2008). RU486 (30 mg/kg body weight) was dissolved in dimethyl sulfoxide (Nacalai Tesque, Kyoto, Japan) and injected subcutaneously immediately after SPS. The dose and administration time of RU486 were decided according to previous studies using RU40555, an analogue of RU486 (Kim et al. 1998; Kohda et al. 2007).

Statistical analysis

All values shown represent mean ± SEM. The results of experiments containing 2 group of rats were analyzed by independent t-test (Experiment 1-2). The results of experiments containing 3 or 4 groups of rats were analyzed by one-way analysis of variance (ANOVA) followed by appropriate post hoc comparisons [Experiment 1-1, 1-3, 2-1, 2-2, 2-3 (fear conditioning)]. In experiment 2-3 (extinction session), freezing in the 3 groups was compared by two-way ANOVA (factors: day, group) for repeated

measures (day) followed by appropriate post hoc comparisons. Results were considered statistically significant at $p < 0.05$.

Results

Experiment 1-1: The effect of SPS on nuclear GR levels in rat hippocampus

We first examined the effect of SPS on the nuclear levels of GR in the rat hippocampus at 1, 2 and 4 hours after SPS. Statistical analysis revealed significant effects of SPS on nuclear GR levels [$F(3,35)=3.71$, $p<0.05$]. Post hoc analysis revealed that whereas the nuclear GR level 2 h after SPS was significantly higher than in the sham group ($p<0.05$), there was no significant difference between SPS at 1 h vs 2 h or at 2 h vs 4 h. There were no significant differences in nuclear GR levels among the 1 h post-SPS group, 4 h-post SPS, and sham groups (Fig. 2).

Experiment 1-2: The effect of SPS on GR binding to the GRE of the Bcl-2 and Bax genes in rat hippocampus

Since there was a significant increase in nuclear GR levels 2 h after SPS, we next performed ChIP-qPCR to examine GR binding to the GREs at the promoters of the Bcl-2 and Bax genes in the rat hippocampus 2 h after SPS. Statistical analysis revealed that GR binding to the GRE of the Bcl-2 gene was significantly higher 2 h after SPS [$t=2.01$, $df=35$, $p<0.05$] (Fig. 3a). Conversely, GR binding to the GRE of the Bax gene did not differ significantly between the SPS and sham groups [$t=2.05$, $df=28$, $p=0.49$] (Fig. 3b).

Experiment 1-3: Effect of SPS on Bcl-2 and Bax mRNA levels

We next performed RT-PCR to examine the effects of SPS on Bcl-2 and Bax mRNA levels 2 h and 4 h after SPS. Statistical analysis revealed a significant effect of SPS on the levels of Bcl-2 mRNA [$F(2,23)=6.19$, $p<0.01$] (Fig. 4a) but not Bax mRNA [$F(2,23)=2.59$, $p=0.10$] (Fig. 4b). Post-hoc analysis revealed that the expression of Bcl-2 mRNA 2 h after SPS was significantly lower compared to the sham group ($p<0.01$), and there were no significant differences between the sham and the 4 h-post SPS groups or between the 2 h-post SPS and 4 h-post SPS groups (Fig. 4a).

Experiment 2-1: Effect of RU486 on SPS-induced alteration of nuclear GR and Bcl-2 gene transcription in rat hippocampus

We next examined the effect of a GR antagonist (RU486) on nuclear GR levels and the levels of Bcl-2 and Bax mRNA 2 h after SPS. With respect to nuclear GR levels, statistical analysis revealed a significant main effect [$F(2,55)=13.88$, $p<0.05$], and post-hoc analysis indicated that nuclear GRs in the SPS group were significantly increased compared with the control group ($p<0.05$) and SPS + RU486 group ($p<0.05$). There was no significant difference between the control group and SPS + RU486 group (Fig. 5a). Statistical analysis also showed significant main effect on Bcl-2 [$F(2,40)=5.18$, $p<0.05$] but not Bax mRNA levels [$F(2,40)=1.59$, $p=0.21$] (Fig. 5b,c). Post-hoc analysis indicated that Bcl-2 mRNA levels were significantly decreased in the SPS group compared with the control ($p<0.05$) and SPS + RU486 groups ($p<0.05$), and there was no significant difference between the control group and SPS + RU486

group (Fig. 5b).

Experiment 2-2: Effect of RU486 on hippocampal apoptosis in SPS rats.

We performed TUNEL staining of the dorsal and ventral portion of the hippocampus (dHPC and vHPC respectively). Statistical analysis revealed a significant main effect for the dHPC [$F(2,53)=5.91$, $p<0.01$], while post-hoc analysis revealed that TUNEL-positive cells in the SPS group were significantly increased compared with the control ($p<0.05$) and SPS + RU486 groups ($p<0.01$), and that there was no significant difference between the control and SPS + RU486 groups (Fig. 6a). Results for the vHPC were almost identical to those for the dHPC; statistical analysis revealed a significant main effect [$F(2,49)=31.49$, $p<0.001$], while post-hoc analysis indicated that hippocampal apoptosis in the SPS group was significantly increased compared with the control ($p<0.01$) and SPS + RU486 groups ($p<0.01$), and that there was no significant difference between the control and SPS + RU486 groups (Fig. 6b).

Experiment 2-3: Effect of RU486 on impaired fear memory extinction in SPS rats.

We next examined the effect of RU486 on impaired fear memory extinction in SPS rats. Fear memory extinction was evaluated using the FCT, and there were no significant differences in freezing during contextual fear conditioning between any groups (Fig. 7). Regarding fear memory extinction, statistical analysis of the data revealed significant main effects of day [$F(1,110)=39.41$, $p<0.01$] and group

[F(2,110)=10.48, p<0.01]. There was no significant day x group interaction [F(2,110)=0.46, p=0.64]. Post hoc analysis revealed no significant differences in freezing time among any of the groups at the extinction training session. At the extinction test session, the freezing time of the SPS group was significantly longer compared with the control (p<0.05) and SPS + RU486 groups (p<0.05), and there was no significant difference between the control group and SPS + RU486 group (Fig. 7).

Discussion

The clinical findings from patients with PTSD, such as enhanced negative feedback of the HPA axis, smaller hippocampal volume, deficits in short-term verbal memory, and impaired fear extinction, have led to the hypothesis that elevated glucocorticoid levels in response to extreme stress trigger the pathophysiological cascade that ultimately leads to functional and morphological changes in the hippocampus. The results of the present study showed that activation of a GR-dependent nuclear cascade by SPS led to an increase in apoptotic cells and a diminished Bcl-2/Bax ratio in the rat hippocampus, whereas administration of RU486 immediately after SPS prevented these apoptotic changes in the hippocampus. Our findings support the involvement of hippocampal abnormalities due to extreme stress in the pathophysiology of PTSD.

Although it is well established that exposure to extreme stress is the primary cause of PTSD, it has remained controversial as to whether elevation of glucocorticoids leads to the pathophysiology of PTSD (Szeszko et al. 2018). In this study, we first examined time-dependent changes in the nuclear GR levels in the hippocampus after SPS, and subsequently found that peak elevation of nuclear GRs occurred at 2 h after SPS. It has been reported that induction of nuclear translocation of GRs in the rat hippocampus by restraint stress is corticosterone-dependent and occur immediately after the administration of stress (Kitchener et al. 2004), and that plasma corticosterone levels are markedly increased following the administration of SPS (Kohda et al. 2007). Based on these findings, since GR is a well-known

transcription factor, it is conceivable that SPS may affect gene transcription in the rat hippocampus through an increase in nuclear GRs in response to increased blood corticosterone levels.

To confirm the possibility that SPS alters gene transcription, we next examined GR binding to the GRE at the promoters of the Bcl-2 and Bax genes by measuring the respective mRNA levels in the rat hippocampus in response to SPS. We found that administration of SPS increased GR binding to the GRE of the Bcl-2 gene but not the Bax gene, and that Bcl-2 mRNA levels were decreased while the levels of Bax mRNA were unchanged. Nuclear GRs regulate gene expression by several modes of action, such as binding to GREs in target genes to activate gene transcription, binding to negative GREs (nGREs) at target genes to inhibit gene transcription, or interacting with other transcription factors (Necela and Cidlowski 2004; Oakley and Cidlowski 2013). Notably, the Bcl-2 gene contains nGRE at its promoter, and its expression is known to be decreased by glucocorticoid treatment (Mocetti et al. 2001; Surjit et al. 2011). These findings further suggest that SPS decreases Bcl-2 gene transcription, but not that of the Bax gene, via activation of GR-dependent nuclear transduction, and consequently lead to a decreased Bcl-2/Bax ratio.

With regard to the reduced Bcl-2/Bax ratio, we further examined whether SPS induced apoptosis in the hippocampus by TUNEL staining, and found an increase in apoptotic cells in the hippocampus of SPS rats. To our knowledge, there are 3 studies examining hippocampal apoptosis and changes in the levels of Bcl-2 and Bax in SPS rats (Li et al. 2010a; Li et al. 2010b; Shafia et al. 2017). Whereas all studies

demonstrated hippocampal apoptosis in SPS rats, there were inconsistent results regarding the hippocampal levels of Bcl-2 and Bax. Results reported by Li and associates (Li et al. 2010b) were in good agreement with our findings; however, they reported upregulation of Bcl-2 and Bax immunoreactivity (Li et al. 2010a). Shafia and associates found increased levels of Bax mRNA with no change in the levels of Bcl-2 mRNA in the hippocampus of SPS rats ((Shafia et al. 2017). Although the reason for this discrepancy is unknown, the different experimental procedures, such as timepoints for measurement, or a difference between mRNA levels and immunoreactivity, may be associated.

In the light of the demonstration of hippocampal apoptosis in SPS rats, it is noteworthy that some meta-analyses have indicated that individuals with PTSD have smaller hippocampal volume than both healthy volunteers and trauma-exposed individuals without PTSD (Nelson and Tumpap 2017; O'Doherty et al. 2015). In addition, the hippocampus is a highly heterogeneous structure and comprises functionally distinct sub-regions having different afferent and efferent connections (Fanselow and Dong 2010; Friedman et al. 2002), and the potential implication of sub-region specific abnormalities has been shown to be relevant to the symptoms of PTSD (Bonne et al. 2008; Keding and Herringa 2015). Together, our findings that SPS significantly increased apoptosis implicates a significant association between GR-dependent nuclear transduction activity and the diverse functional disturbances in PTSD.

Notably, the transiently increased levels of nuclear GR, decreased levels of Bcl-2 mRNA, and increase in apoptotic cells in the rat hippocampus by SPS were markedly prevented by administration of the GR

antagonist RU486 immediately after SPS. Treatment with 30 mg/kg of RU486 immediately after SPS inhibited the activation of GR-dependent nuclear transduction by SPS, although RU486 at a dose of 20 mg/kg was reported to act as a partial agonist (Spiga et al. 2011). Thus, in the present study, the ability of RU486 to prevent the decrease in Bcl-2 expression and increase in cellular apoptosis in response to SPS may be, at least in part, mediated by inhibition of the nuclear translocation of GRs.

Since administration of RU486 immediately after SPS inhibited SPS-induced apoptotic signal transduction in the rat hippocampus, it is plausible that RU486 may have a preventive effect on impaired fear extinction in SPS rats. In fact, our study did find an inhibitory effect of this compound on impaired fear extinction in SPS rats. In line with the validity of SPS as an animal model of PTSD, a series of our studies and those by other researchers have demonstrated impaired fear extinction in SPS rats. In particular, the present study and a recent study by (Shafia et al. 2017) demonstrated both an increase in apoptotic cells in the vHPC and impaired fear extinction in SPS rats. It is well known that extinction is an active leaning process but that it does not erase the traumatic memory. In this context, it is likely that impaired extinction is due to impaired spatial leaning mediated by hippocampal apoptosis. However, since both (Shafia et al. 2017) and (Yamamoto et al. 2008) reported that there was no significant difference in freezing time 24 h after fear conditioning (the first day of extinction training) between SPS and sham rats, it is unlikely that the impaired spatial learning by hippocampal apoptosis is involved in the impairment of fear extinction. Furthermore, we previously suggested that decreased levels of NR2B

mRNA and protein in the hippocampus may be closely associated with impaired fear extinction in SPS rats. Collectively, it is plausible that not only GR-induced apoptosis but also decreased NR2B in the hippocampus may contribute to impaired fear extinction in SPS rats (Matsumoto et al. 2013). Since it has been recently suggested that impaired fear extinction is one of the hallmark symptoms of PTSD, further studies elucidating the precise mechanism of impaired fear extinction are necessary to develop an effective prevention strategy for PTSD.

The efficacy of the glucocorticoid intervention on the prevention and treatment of PTSD is inconsistent across both human and animal studies. While administration of hydrocortisone is reported to be useful for the prevention of PTSD (Amos et al. 2014; Birur et al. 2017), GR antagonists and corticotropine-releasing factor type 1 antagonists are examined whether these drugs are effective in the treatment of PTSD (Dunlop et al. 2017; Golier et al. 2012). With regard to the preclinical studies, Rao and associates demonstrated that administration of corticosterone prior to restraint stress prevented the delayed increase in anxiety-like behavior (Rao et al. 2012). In addition, Keller and associates showed that administration of GR antagonist during extinction training exacerbated extinction retention deficits of SPS rats (Keller et al. 2015). In contrast, Pitman and associates have shown that systemic mifepristone blocked the reconsolidation of cue-conditioned fear in contextual fear conditioning paradigm in rats (Pitman et al. 2011). Based on these controversial findings, it is plausible that the relationship between the glucocorticoid intervention and status of GR-mediated signal may play a role in the effect of

glucocorticoids on the development of PTSD. In particular, administration of GR antagonist prevented the increase in the nuclear GRs by SPS and alleviated the impaired fear extinction in SPS rats in this study. Although further studies are required, these evidences support the potential of hippocampal GR inhibition immediately after stress for prevention of the development of PTSD.

With regard to fear memory extinction, it is well known that amygdala also plays an important role. Although we did not examine the nuclear GRs in the amygdala, it was reported that FC enhanced GR internalization and SPS attenuated the enhanced GR internalization in response to FC (Moulton et al. 2018). In this context, further studies examining the nuclear GR in the amygdala of SPS rats are required to elucidate the involvement of GR-mediated signal on the development of impaired fear extinction.

In summary, exposure to SPS increased GR nuclear translocation and subsequent GR binding to the GRE of the Bcl-2 gene, leading to a decrease in Bcl-2 mRNA levels without any change in Bax expression. Thus, an imbalance in apoptotic proteins (Bcl-2/Bax ratio) might lead to increased hippocampal apoptosis 1 week after SPS. Systemic administration of the GR antagonist RU486 immediately after SPS prevented activation of the glucocorticoid cascade and hippocampal apoptosis in SPS rats. The preventive effect of RU486 on impaired fear memory extinction in SPS rats may be, at least in part, due to inhibition of hippocampal apoptosis. Based on these findings, we postulate that activation of hippocampal GRs in response to severe stress triggers the pathophysiological cascade that leads to impaired fear extinction, and the inhibition of hippocampal GR immediately after stress could be useful

for preventing the development of PTSD.

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Figure Captions

Fig. 1 Timelines for (a, b) biochemical and (c) behavioral tests**.**

(a) Number signs 1-3 indicate the timepoints for hippocampal tissue sampling (#1, Nuclear GR, #2 nuclear GR, chromatin immunoprecipitation, real-time RT-PCR, #3 nuclear GR, real-time RT-PCR)

(b) Number signs indicate the timepoints for hippocampal tissue sampling (#1, nuclear GR, real-time RT-PCR, #2 TUNEL staining)

(c) FC, fear conditioning; Ext Tr, extinction training; Ext Te, extinction test

Fig. 2 Effect of SPS on nuclear translocation of GRs.

Representative blots for GR and TATA binding protein (TBP) are shown in upper panel. Data are expressed as the ratio of expression of GR to that of TBP (GR/TBP) and shown as the mean ± SEM of the 8 to 10 rats per group. Nuclear GR levels were significantly increased at 2 h after SPS. *$p<0.05$; one-way ANOVA with Tukey post-hoc analysis

Fig. 3 Effect of SPS on GR binding to the GRE of (a) Bcl-2 gene and (b) Bax gene.

Data are expressed as the ratio of target sequence (Bcl-2 or Bax) to that of input DNA (target sequence/input DNA) and shown as the mean ± SEM of 16 to 20 rats per group. (a) Binding to the GRE of the Bcl-2 gene was significantly increased 2 h after SPS. (b) There was no significant difference in

binding to the GRE of the Bax gene compared with the sham group. *p<0.05; independent t test

Fig. 4 Effect of SPS on (a) Bcl-2 and (b) Bax mRNA expression.

Data are expressed as the ratio of target gene (Bcl-2 or Bax) expression to that of GAPDH (target gene/GAPDH) and shown as the mean ± SEM of 8 to 10 rats per group. (a) Bcl-2 mRNA expression was significantly decreased 2 h after SPS. (b) There was no significant difference in Bax mRNA expression compared with the sham group. **p<0.01; one-way ANOVA with Tukey post-hoc analysis

Fig. 5 Effects of RU486 on (a) GR nuclear translocation (b) Bcl-2 mRNA expression (c) Bax mRNA expression.

(a) Representative blots for GR and TATA binding protein (TBP) are shown in the upper panel. Data are expressed as the ratio of GR expression to that of TBP (GR/TBP) and shown as the mean ± SEM of 19 to 20 rats per group. Nuclear GR levels were significantly increased in the SPS group compared with the sham and SPS+RU486 groups. *p<0.05; one-way ANOVA with Tukey post-hoc analysis

(b, c) Data are expressed as the ratio of target gene (Bcl-2 or Bax) expression to that of GAPDH (target gene/GAPDH) and shown as the mean ± SEM of 13 to 16 rats per group (b) Bcl-2 mRNA expression was significantly decreased in the SPS group compared with the sham group and SPS+RU486 group. (c) There were no significant differences in Bax mRNA expressions. *p<0.05; one-way ANOVA with Tukey

post-hoc analysis

Fig. 6 Effect of RU486 on hippocampal apoptosis in SPS rats.

Representative images of TUNEL staining in the hippocampus are shown in the upper panel. (a) dorsal hippocampus (dHPC), (b) ventral hippocampus (vHPC), Scale bar = 500 μm.

Data are expressed as the ratio of TUNEL-positive cells to total cells (TUNEL-positive cells/total cells) and shown as the mean ± SEM of 16 to 20 sections per group (4 sections per rat). Hippocampal apoptosis in the SPS group was significantly increased compared with the sham and SPS+RU486 groups both in the (a) dHPC and (b) vHPC. *$p<0.05$, **$p<0.01$; one-way ANOVA with Tukey post-hoc analysis

Fig. 7 Effect of RU486 on impaired fear memory extinction in SPS rats.

Data are expressed as mean ± SEM of 16 to 21 rats per group. At the fear conditioning and extinction training session, there were no significant differences among the groups. At the extinction test session, the SPS group showed impaired fear memory extinction compared with the sham group, and the impaired fear memory extinction was inhibited by administration of RU486. *$p<0.05$; two-way ANOVA with Tukey post-hoc analysis

Table 1. Primer and probe sequences used for real-time PCR (for quantification of GR-immunoprecipitated DNA)

B-cell leukemia/	Forward primer	5'-TCCCTGAGCAAGTAGATGCAGTT-3'
Lymphoma 2	Reverse primer	5'-TCTATTCAGAATGGAGGCTCTATCC-3'
(Bcl-2)	TaqMan MGB probe	5'-CCTCGGCCGAGACT-3'
Bcl-2 associated	Forward primer	5'-CTCGTAGGTCTCCGATAGTTCCTT-3'
X(Bax)	Reverse primer	5'-AAGGAAACGGAGGCATCAGTAC-3'
	TaqMan MGB probe	5'-CTCCAAATGGCCTGCAGA-3'

Minor groove binder (MGB) is a Tm enhancer that increases sequence specificity.
FAM was used as quencher dye, TAMRA was used as reporter dye.

Table 2. Primer and probe used for RT PCR

	GenBank accession Number	Applied biosystems assay ID
B-cell leukemia/lymphoma 2 (Bcl-2)	NM 016993	Rn99999125_m1
Bcl-2-associated X (Bax)	NM 017059	Rn01480161_g1

Figure 1.

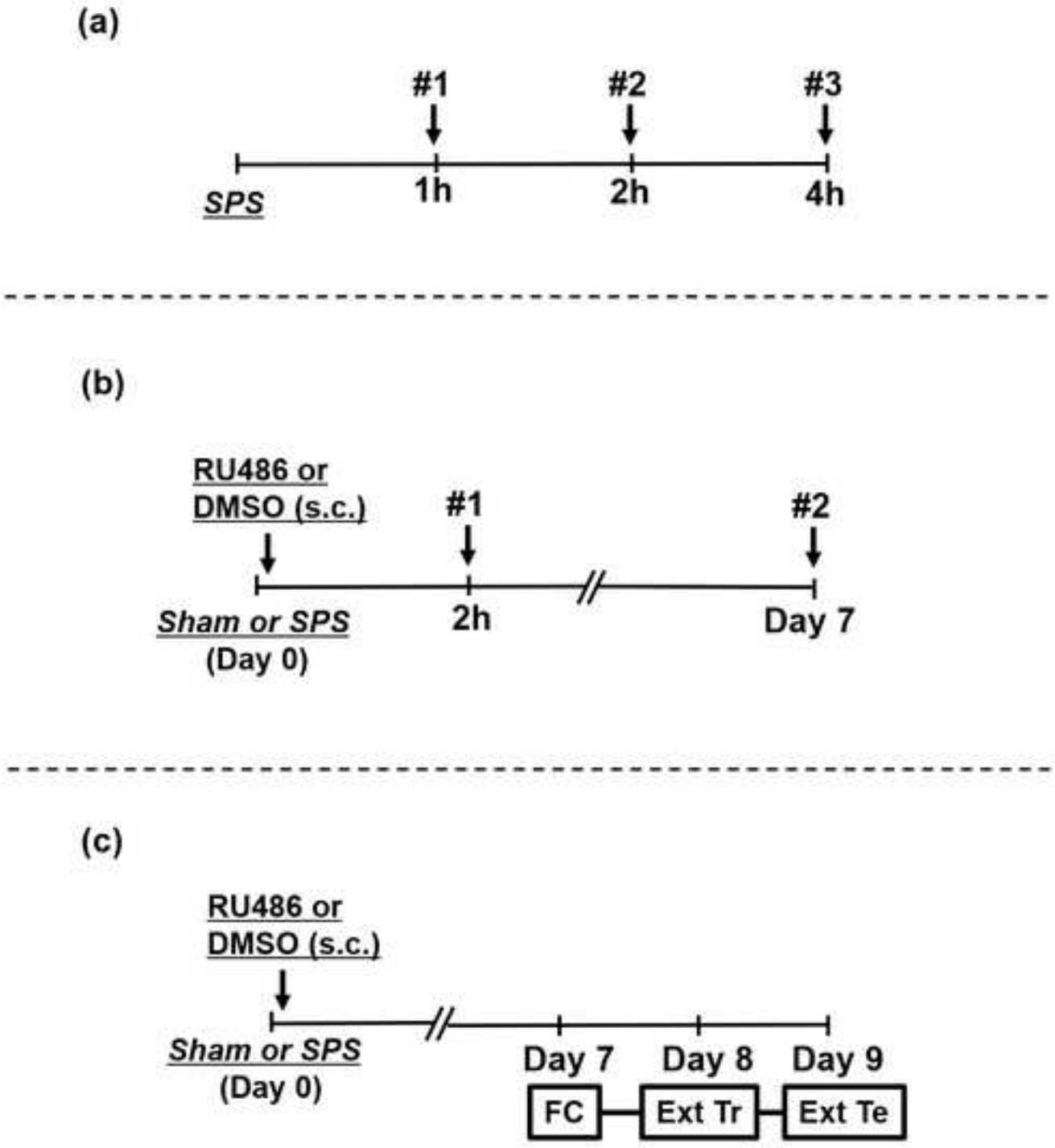

Figure 2.

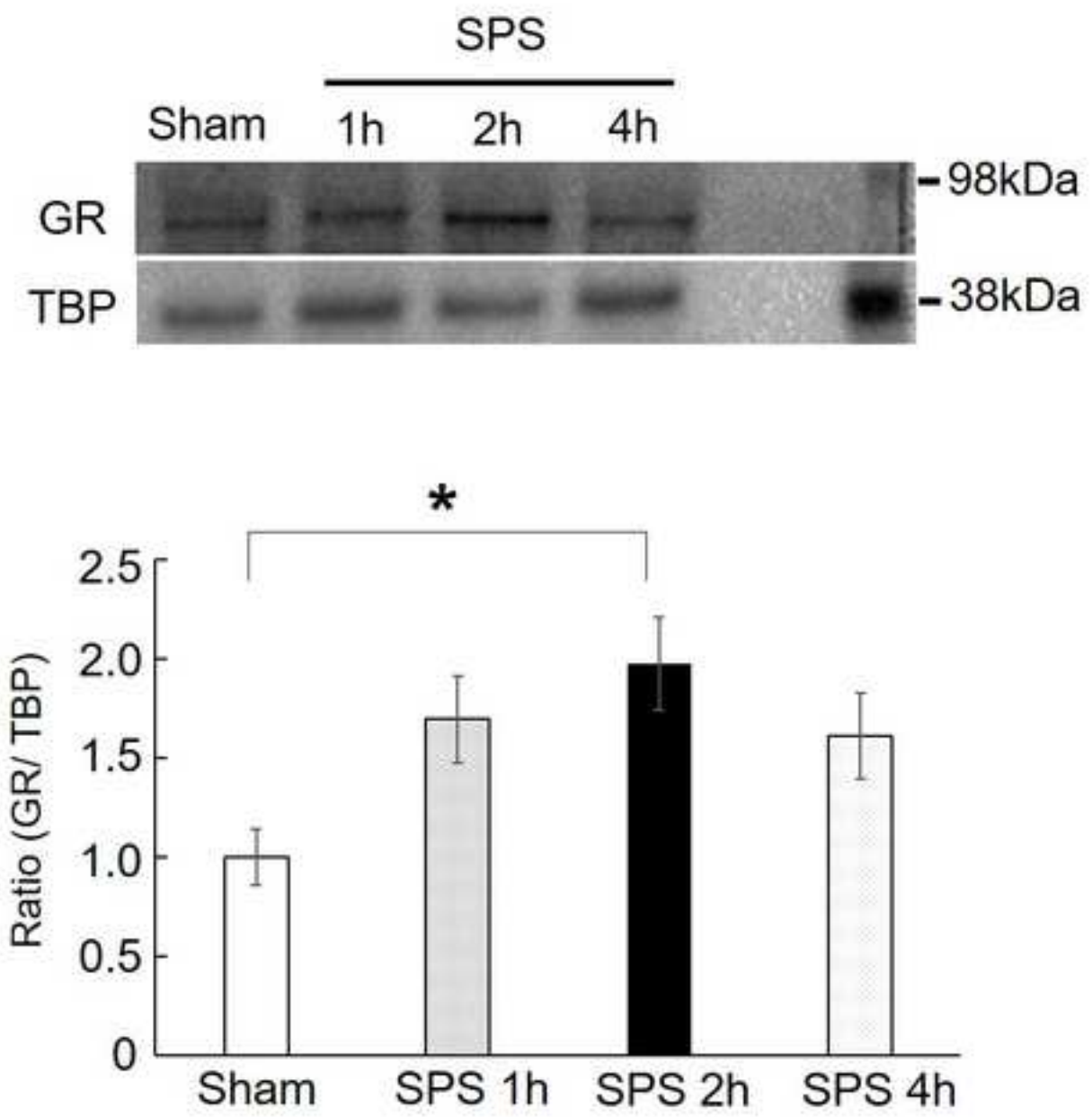

Figure 3.

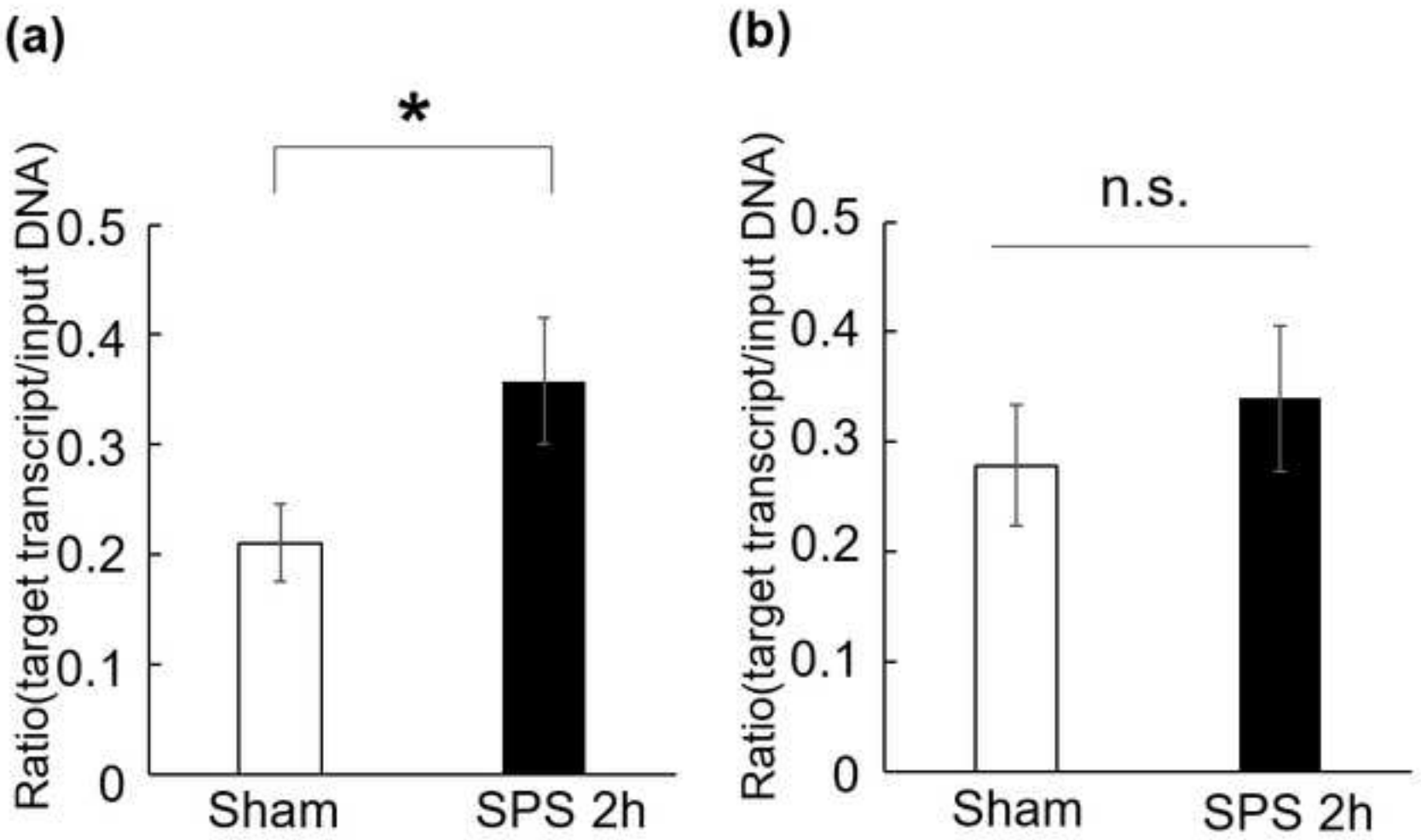

Figure 4.

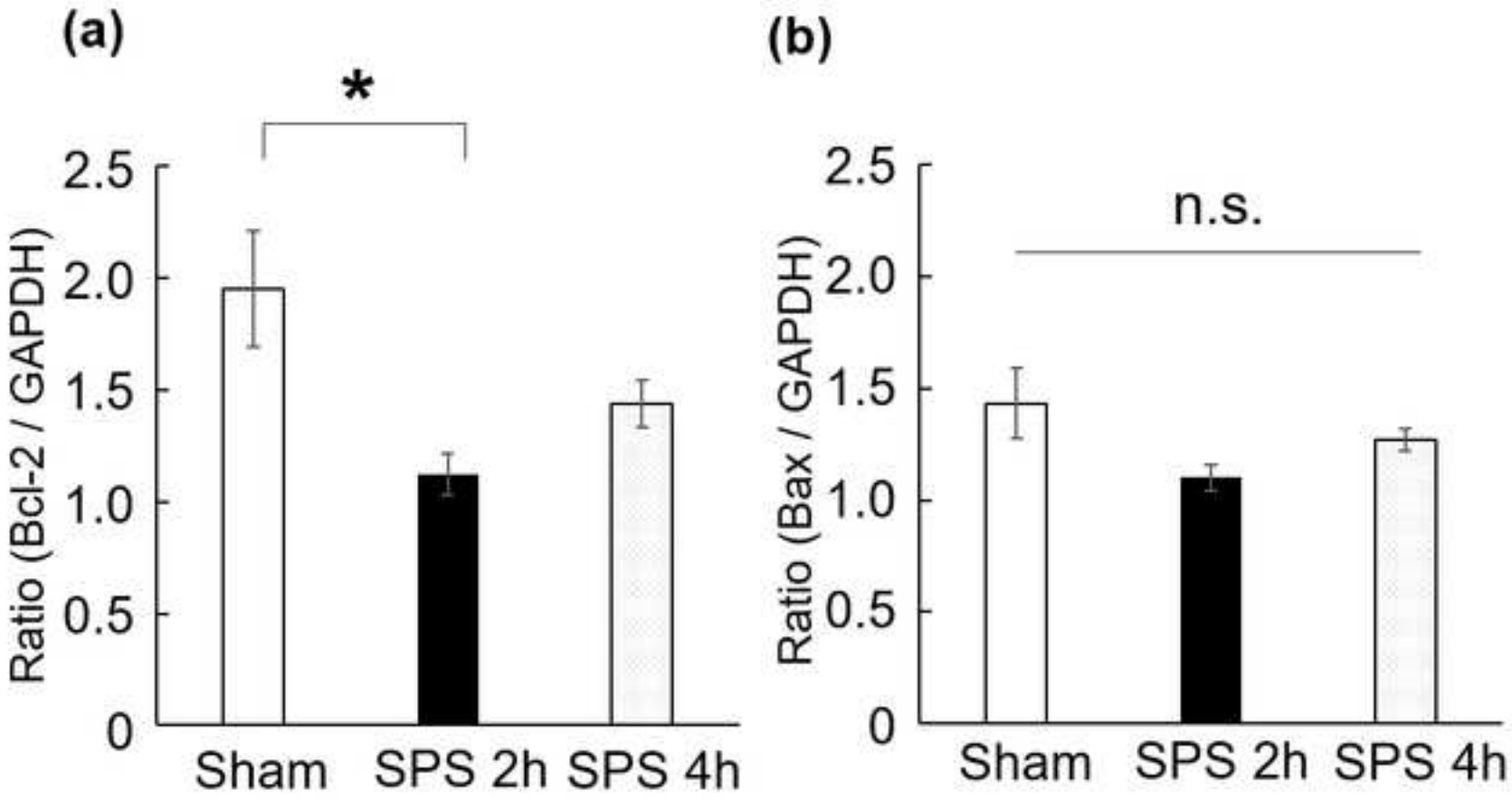

Figure 5.

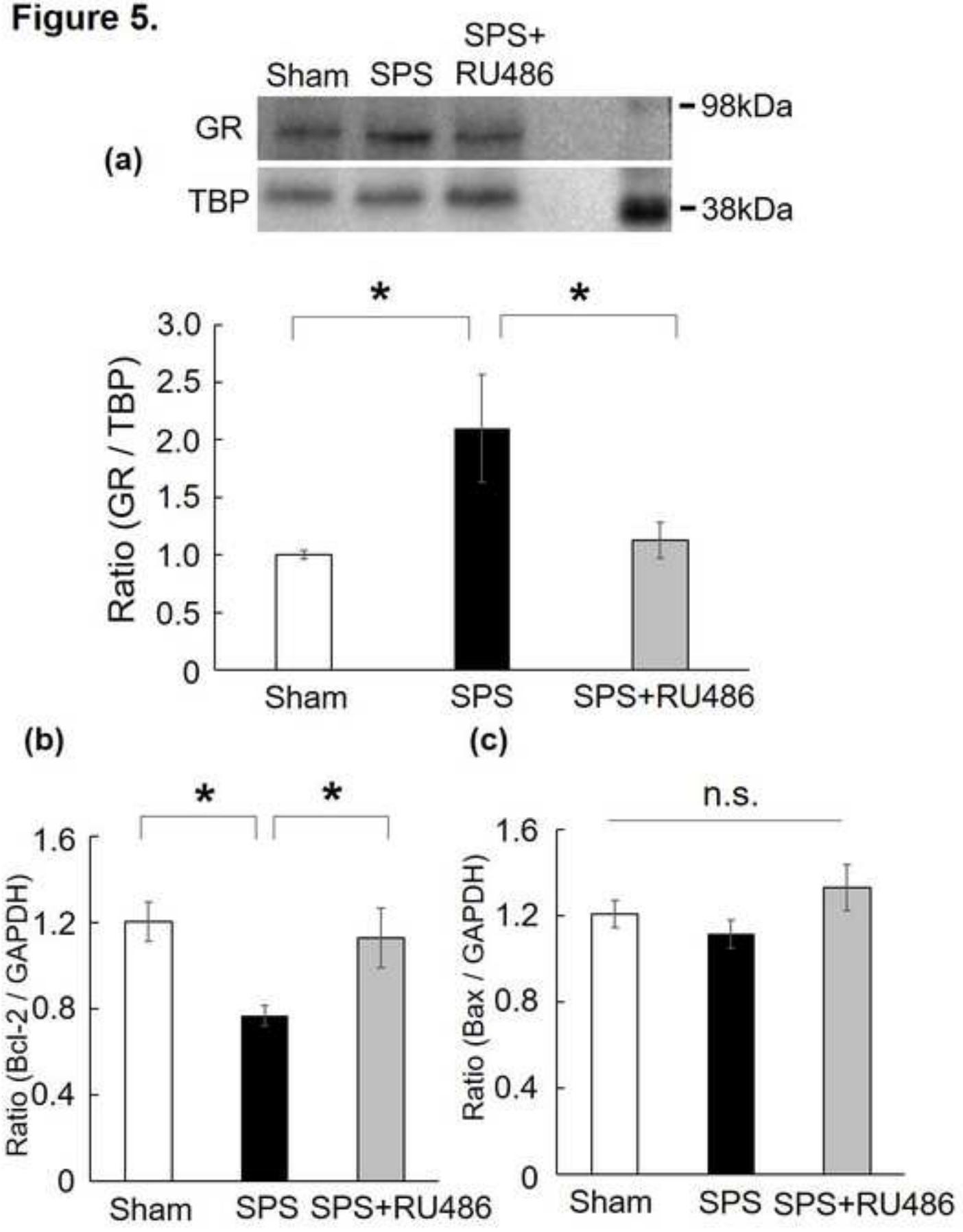

Figure 6

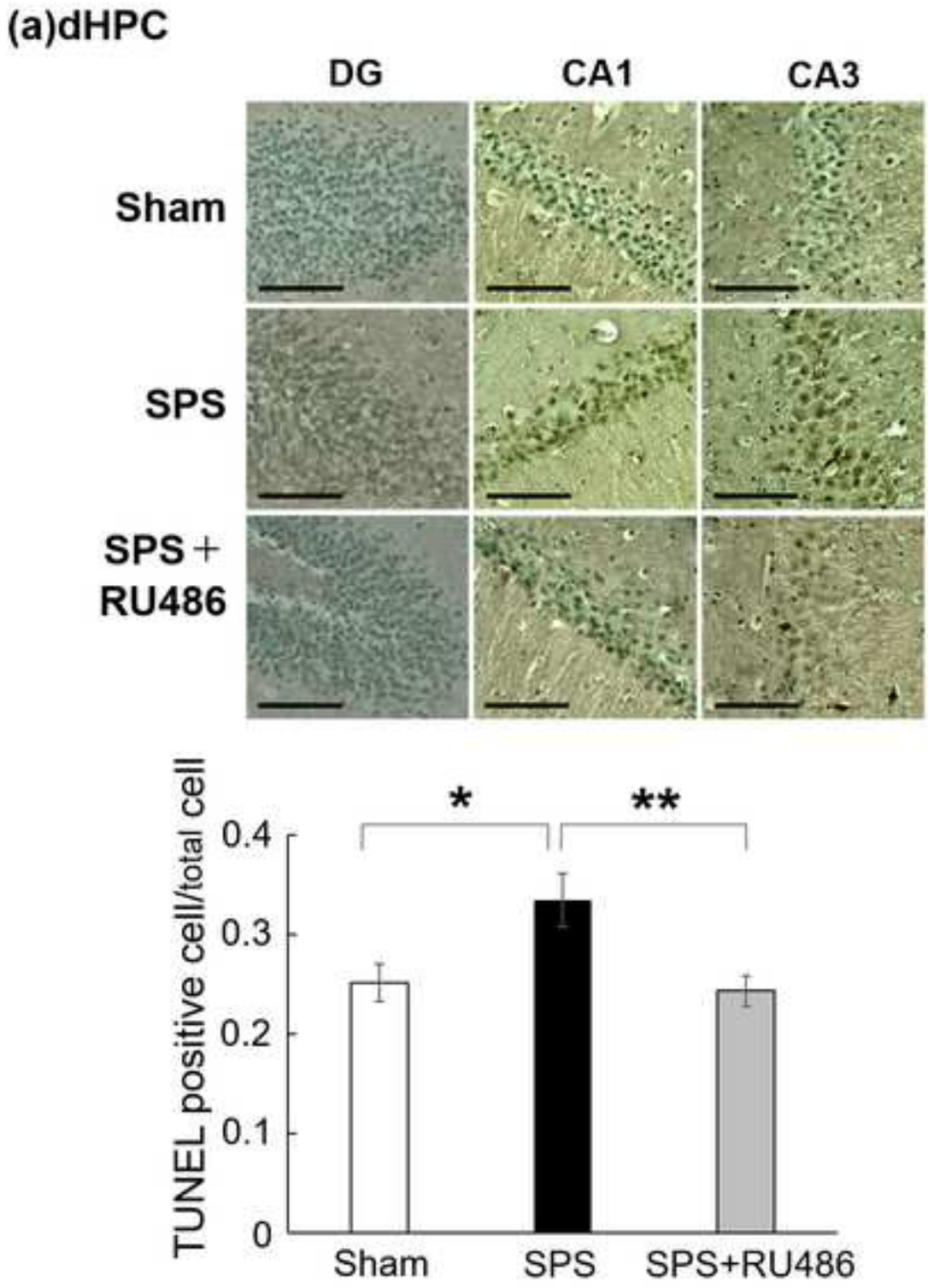

Figure 6

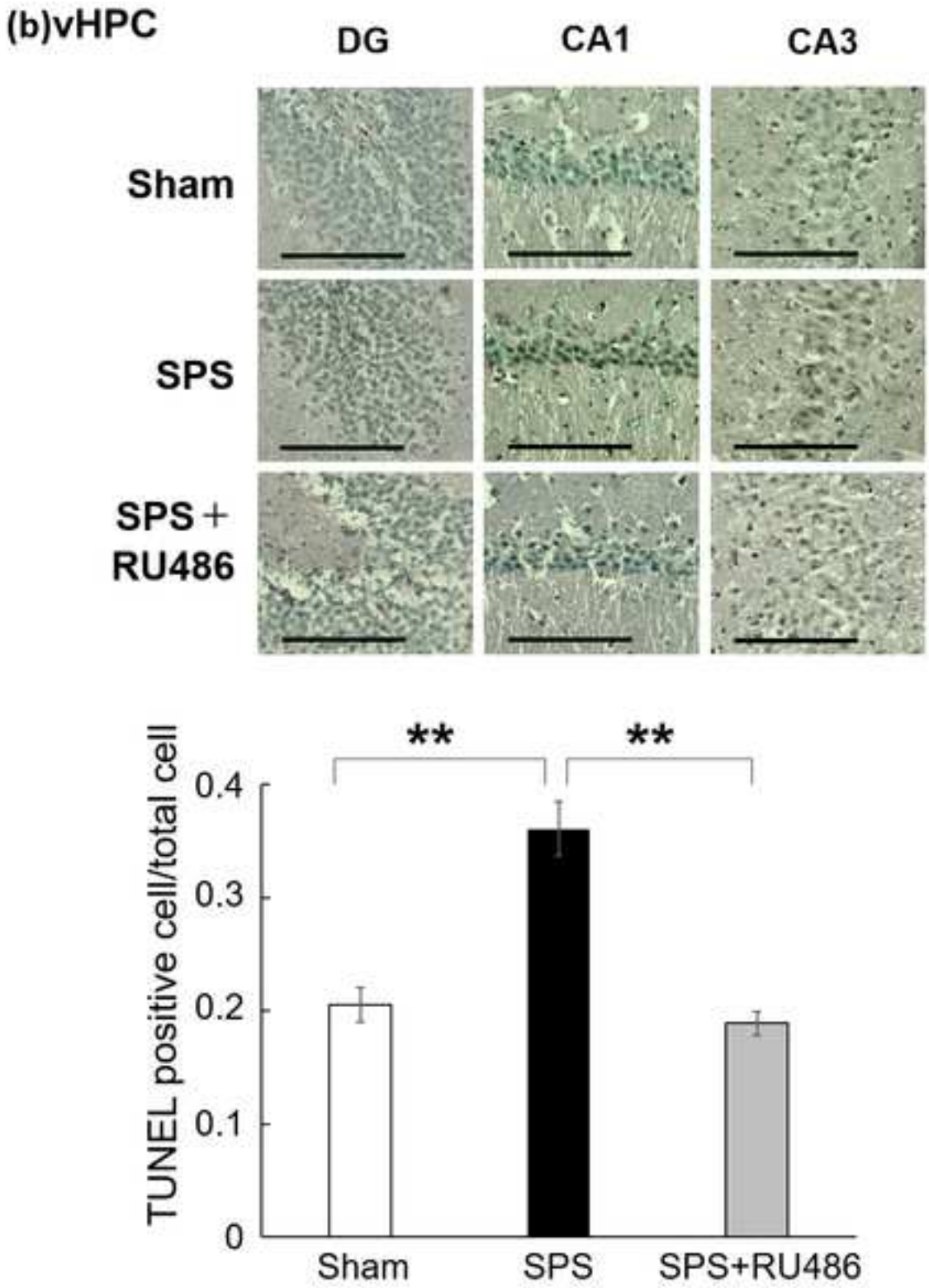

Figure 7