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Original Article

Intact Recognition Memory and Altered Hippocampal Glucocorticoid Receptor Signaling in Fkbp5-deficient Mice Following Acute Uncontrollable Stress

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The FK506 binding protein 5 (FKBP5) is a co-chaperone that regulates the activity of the glucocorticoid receptor (GR) and has been reported to mediate stress resilience. This study aimed to determine the effects of *Fkbp5* deletion on acute stress-induced recognition memory impairment and hippocampal GR signaling. Wild-type and *Fkbp5*-knockout mice were subjected to acute uncontrollable stress induced by restraint and electrical tail shock. First, we assessed the cognitive status of mice using a novel object recognition task. Next, we measured plasma corticosterone, GR levels, and the levels of GR phosphorylation at serine 211 in the hippocampus. Wild-type mice exhibited stress-induced memory impairments, whereas *Fkbp5*-knockout mice did not. Plasma corticosterone and GR levels did not differ between the non-stressed wild-type and *Fkbp5*-knockout mice, but the levels of phosphorylated GR were lower in *Fkbp5*-knockout mice than in wild-type mice. Wild-type and *Fkbp5*-knockout mice showed increased nuclear GR levels following stress, indicating GR translocation. However, cytosolic phosphorylated GR levels were lower in the hippocampi of *Fkbp5*-knockout mice following stress than in those of wild-type mice. These results suggest that FKBP5 deficiency increases resilience to acute stress by altering GR signaling.

Key words: FKBP5, Glucocorticoid receptor, Hippocampus, Memory, Mice

INTRODUCTION

The hippocampus is vulnerable to uncontrollable stress [1-3] and is enriched with glucocorticoid receptors (GR) [4-6]. Accordingly, animals that experience acute uncontrollable stress perform poorly in hippocampal-dependent memory tasks, such as the hidden platform water maze and novel object recognition [3, 7-9]. Furthermore, GR signaling and expression levels of FK506-binding protein 5 (FKBP5) are altered in the hippocampus during acute or chronic stress [10-13]. Recently, levels of GR phosphorylation at serine 203 and serine 211 were reported to increase in the hippocampus of rats with chronic subcutaneous corticosterone injections [14].

Several studies have reported that FKBP5 is a co-chaperone that regulates GR activity, which in turn mediates stress resilience [13, 15, 16]. For example, *Fkbp5*-deficient mice showed active coping behavior and reduced hypothalamus-pituitary-adrenal axis reactivity to stressors. Furthermore, hippocampal GR protein levels were higher in *Fkbp5*-deficient mice than in wild-type mice eight days after receiving the 60-min restraint and forced swimming test [13]. In addition, *Fkbp5*-deficient mice and mice with FKBP5 pharmacological inhibition did not exhibit depression-like

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behavior following chronic stress [17-19]. However, no study has examined the effect of FKBP5 deficiency on acute stress-induced memory impairment and hippocampal GR signaling.

This study aimed to determine the effects of *Fkbp5* deletion on acute stress-induced recognition memory impairment and alterations in hippocampal GR signaling. Under baseline conditions, the corticosterone and hippocampal total GR levels of the *Fkbp5*-knockout mice showed no differences from the wild-type controls, but the total protein levels of the phosphorylated GR at serine 211 (pGR S211), a key regulator of receptor transcriptional activation and repression [20], were lower. Wild-type and *Fkbp5*-knockout mice were subjected to acute uncontrollable stress, and their recognition memory was assessed. The recognition memory of wild-type mice, not *Fkbp5*-knockout mice, was impaired. In addition, plasma corticosterone levels were lower in *Fkbp5*-knockout mice than in the wild-type mice following stress. Finally, we evaluated the status of hippocampal GR in *Fkbp5*-knockout mice following stress. Stress-induced GR nuclear translocation occurred in wild-type and *Fkbp5*-knockout mice. However, cytosolic pGR S211 levels were lower in the hippocampi of *Fkbp5*-knockout mice following stress than in those of wild-type mice. These results suggest that FKBP5 deficiency increases resilience to acute uncontrollable stress by altering GR signaling.

MATERIALS AND METHODS

Animals

Wild-type and *Fkbp5*-knockout mice on a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). Three-to four-month-old wild-type (male, n=32; female, n=34) and *Fkbp5*-knockout mice (male, n=35; female, n=35) were used in this study. The mice were kept under a 12 h : 12 h light-dark cycle (lights on at 08:00 h) in a temperature (22±1℃) and humidity (50±10%) controlled vivarium. Food and water were provided ad libitum. All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Konkuk University (KU16194, 17194, and 21149).

Genotyping

Tail sampling was performed in three-week-old mice and then stored in a microcentrifuge tube (see Fig. 1). A tail mix buffer (0.5% sodium dodecyl sulfate [SDS], 0.1 M NaCl, 50 mM Tris [pH 8.0], 2 mM EDTA), and proteinase K (10 mg/ml) were added to the microcentrifuge tube containing the tail and incubated overnight in a 56℃ water bath. Following overnight incubation, 8 M potassium acetate (75 μl) and chloroform (400 μl) were added to the microcentrifuge tube and centrifuged (Smart R17, Hanil Science, South Korea) for 15 min, at 4℃, 9358×g. After centrifugation, the supernatant was transferred to a new tube, and 100% ethanol (1 ml) was added. Another centrifugation was performed (4℃, 18,341×g, 5 min), the supernatant was discarded, and 70% ethanol (700 μl) was added to dissolve the pellet. One last centrifugation (4℃, 18,341×g, 5 min) was performed, the supernatant was discarded, and the resulting deoxyribonucleic acid (DNA) pellet was stored in Tris-acetate-EDTA (TAE) buffer (100 μl). Polymerase chain reaction (PCR) was performed using 2× Biomix (Meridian Bioscience, Cincinnati, OH, USA), DNA, and primers. A common forward primer (5'-AAA GGA CAA TGA CTA CTG ATG AGG-3') and two reverse primers (5'-AAG GAG GGG TTC TTT TGA GG-3' and 5'-GTT GCA CCA CAG ATG AAA CG-3') were used for wild-type and *Fkbp5*-knockout mice. The band size for the wild-type mouse was 363 base pairs (bp), and the band size of the *Fkbp5*-knockout mouse was 510 bp. The mice with both bands were heterozygous (Fig. 1). After PCR was completed, electrophoresis was performed on a 1.5% agarose gel. After electrophoresis, the bands were confirmed using ImageQuant LAS 500 (GE Healthcare, Chicago, IL, USA).

Corticosterone assay and collection of the hippocampal tissues

Blood and hippocampal tissues were collected from wild-type and *Fkbp5*-knockout mice under basal and non-stressed conditions. Another cohort of mice was sacrificed immediately after stress treatment and samples were collected. All procedures were performed between 08:00 and 11:00 h during the diurnal rhythm when basal corticosterone levels were low. Blood plasma was separated by centrifugation (94×g, 15 min) at 4℃ and stored at -70℃. Plasma corticosterone concentrations were measured using a corticosterone rat/mouse enzyme-linked immunosorbent assay (ELISA) kit (IBL-America, Minneapolis, MN, USA).

Stress procedure

Before the stress procedure, all mice were familiarized with the object recognition box for five days. The stress procedure consisted of restraining the animals for 60-min in a cone-shaped plastic bag and administering 60 intermittent tail-shocks (0.45 mA intensity; 1 s duration; 30 to 90 s inter-shock interval), through copper electrodes attached to their tails. The control animals were left undisturbed in their cages. At the end of the stress procedure, the mice were placed back in their home cages for 1 h of recovery before beginning the object recognition task (Fig. 1).

Novel object recognition (NOR) task

This study employed a modified version of the spontaneous

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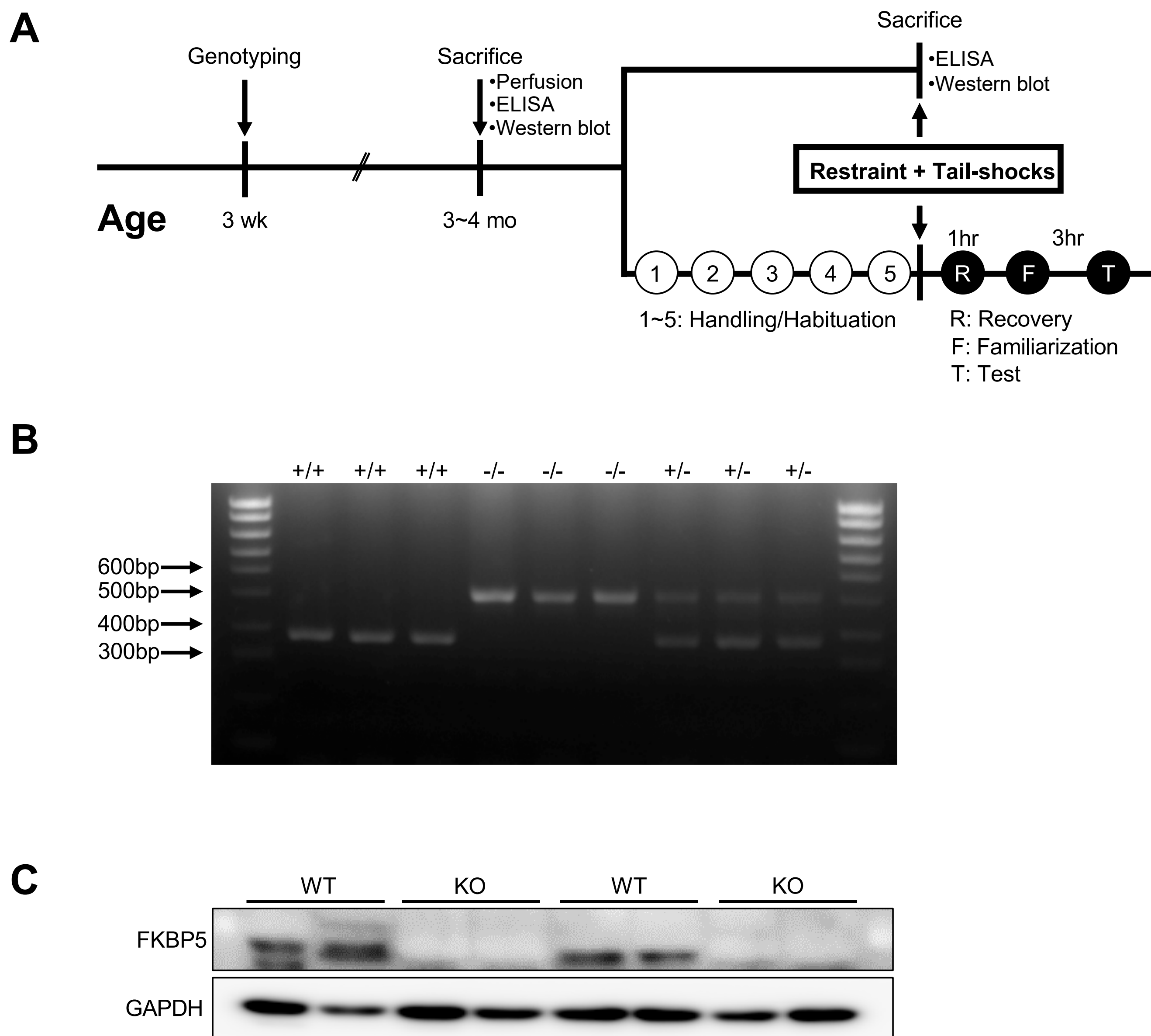

Fig. 1. Experimental procedure and genotyping of wild-type and *Fkbp5*-knockout mice. (A) Schematic diagram of the experimental procedure, including the novel object recognition task. (B) Agarose gel obtained for *Fkbp5* genotyping. The wild-type (+/+) and *Fkbp5*-knockout (-/-) band sizes were 363 bp and 510 bp, respectively. Two bands indicate a heterozygote (+/-). (C) Representative western blot images of brain tissue from wild-type (WT) and knockout (KO) mice.

object recognition task [8, 21-23], initially developed by Ennaceur and Delacour [24], which exploits rodents' natural tendency to explore novel stimuli. The task was conducted inside a black open-field square box (27×34×26.5 cm) with a constant masking white noise of 70 dB source. The box was wiped with 70% ethanol between animals. Mice were handled for 5-min daily and were placed inside the box for 10 min daily to familiarize themselves with transportation and the empty arena for five days. On the sixth day, 1 h after the stress procedure, the animals underwent successive sessions of familiarization, delay, and recognition memory tests (Fig. 1). The familiarization phase consisted of 1-minute of re-habituation in the open-field box and a brief transfer of the animals to their home cages, while two identical objects were placed at the two corners of the box. The animals were then placed back in the box where they remained until they cumulatively explored the objects for 20 s. Upon reaching the 20 s of object exploration criterion (variable time in the arena), animals were placed back in their home cages for 3 h, until the test phase. During the delay period, an identical object to that placed in the familiarization phase (but not scent-marked) and a novel object were placed in the same

two corners (counterbalanced between animals) as in the familiarization phase. After the delay, animals were reintroduced to the box and remained there until they accumulated a total of 20 s of exploration of the two different objects. Exploration behavior was quantified using a computer-assisted scoring program (QBASIC), where manual keystrokes on a computer keyboard recorded the duration and frequency of object exploration [8]. Exploration was only scored when the snout of the mouse was directly facing and sniffing the objects, and not when another body part contacted the objects, such as the forepaws on the object and rearing. During the familiarization and test phases, one mouse did not meet the 20-s exploration criterion within the 10-min allotted time in the open-field box and was excluded from the study. Preference for the novel object was computed as the time spent exploring the novel object rather than the familiar object. Computer scoring of the behavior was performed by Y.-J.J. and Y.-S.J., who matched 87% of the joint observations based on randomly selected familiarization and test sessions from ten mice.

Brain preparations

All mice were deeply anesthetized with isoflurane and perfused with ice-cold 0.01 M phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in 0.01 M PBS for histological analysis. Brains were removed immediately and placed in 4% paraformaldehyde in 0.01 M PBS at 4℃ for 48 h. After fixation, the brain samples were embedded in 30% sucrose in 0.01 M PBS until the brain sunk down to the bottom of the glass jar. Each brain sample was quickly frozen using dry ice and stored at -70℃. Before staining, brain samples were embedded with Tissue-Tekⓡ (Sakura, Torrance, CA, USA), sliced into 30-µm coronal sections, and stored in cryoprotectant (30% ethylene glycol, 25% glycerol, 25% 0.1 M phosphate buffer, and 20% distilled water). For total protein extracts, individual tissue samples were homogenized in ice-cold lysis buffer consisting of 20 mM Tris (pH 7.5), 5% glycerol, 1.5 mM EDTA, 40 mM KCl, 0.5 mM dithiothreitol, and protease inhibitors. The homogenates were then centrifuged at 18,341×g for 1 h at 4℃, and the supernatant was harvested and stored at -80℃ until further analysis.

Preparation of cytosolic and nuclear extracts

Protein extraction was performed using a ReadyPrep™ Protein Extraction Kit (Bio-Rad, Hercules, CA, USA). Hippocampi were dissected and homogenized with cytoplasmic protein extraction buffer (CPEB) in a glass tissue grinder (Radnoti, Covina, CA, USA). After centrifugation at 1,000×g for 10 min, at 4℃, the resulting supernatant was used as the cytoplasmic protein fraction. CPEB was added to extract nuclei from the pellet, and the mixture was centrifuged at 1,000×g for 10 min, at 4℃. Protein solubilization buffer was added to the mixture, which was then centrifuged at 16,000×g for 20 min, at 24℃. The resulting supernatant was used as the nucleic protein fraction.

Western blotting

Protein concentrations in the total cell extracts and cytosolic and nuclear fractions were determined using a bicinchoninic acid assay. Proteins in the extracts were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane using a Mini Trans-Blot Cell (Bio-Rad). After blocking, the membranes were incubated with polyclonal anti-Fkbp5 (1:1,000; Origene, Rockville, MD, USA), polyclonal anti-GR (1:1,000; Santa Cruz, Dallas, TX, USA), polyclonal anti-pGR S211 (1:1,000; Cell Signaling, Danvers, MA, USA), anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5,000; Cell Signaling) or anti-actin antibody (1:5,000, Sigma, St. Louis, MO 68178), followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoreactive proteins were visualized using an enhanced chemiluminescence system (GE Healthcare) and images were captured using an ImageQuant LAS 500 CCD camera (GE Healthcare). The protein band intensity of GR and pGR was normalized to the intensity of the GAPDH band or actin band using Image J software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997~2018).

Immunohistochemistry

Brain sections were washed in PBS containing 0.3% Triton X-100 (PBS-T) and then incubated in a blocking solution (10% fetal horse serum and 0.3% Triton X-100 in PBS) for 2 h at room temperature. Next, they were incubated overnight with a cocktail of primary antibody solution (guinea pig anti-NeuN antibody, 1:1,000, Merck Millipore, Burlington, MA, USA; rabbit anti-pGR antibody, 1:200, Cell Signaling), which contained 1.5% horse serum in PBS-T. The sections were then washed in PBS-T and incubated for 1 h at room temperature with a cocktail of secondary antibody solution (Alexa Fluor 488 conjugated donkey anti-rabbit antibody, 1:200, A21206, Invitrogen, Waltham, MA, USA; Alexa Fluor 633 conjugated donkey anti-guinea pig, 1:200, A21105, Invitrogen), which contained 1.5% horse serum in PBS-T. Subsequently, sections were mounted on resin-coated slides, dried for 1 h, and finally coverslips were mounted with ProLong Gold Antifade Mountant (Invitrogen). Images were obtained using a confocal microscope (LSM 800, Carl Zeiss, Oberkochen, Germany). At least six sections were selected per animal.

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Data analysis

The time spent exploring the two objects during the familiarization and test phases of the NOR task was evaluated in the same animals. Thus, we utilized a one-sample t-test (two-tailed significance) to analyze the behavioral data [8, 22], where a test value setting of 10 s denoted no object preference. The preference in the NOR task, corticosterone levels, total GR, and pGR levels were analyzed using analysis of variance (ANOVA) and an independent t-test. Post hoc analyses were conducted using Fisher's least significant difference test, if necessary. All data are expressed as boxplots or means±standard error of the mean. The alpha level was set to 0.05. SPSS Statistics 25 (IBM, Armonk, NY, USA) and Prism 9 software (GraphPad Software, San Diego, CA, USA) were used for statistical analyses and graphical figures, respectively.

RESULTS

Intact recognition memory in Fkbp5-knockout mice following uncontrollable stress

Thirty-six mice from all groups equally explored the two identical objects placed at the left and right sides (L and R) in the open-field box during the familiarization phase (all $p>0.05$), indicating no left-right side bias against the object location (Fig. 2A). All groups required a similar amount of time to reach the 20-s exploration criterion ($F_{3,32}<0.7$, $p>0.77$) in the familiarization phase, suggesting that the behavioral impairment observed in this experiment was not due to alterations in exploratory or locomotor activity. During the test phase (Fig. 2A), significantly more time was spent exploring the novel object than the familiar object in the non-stressed wild-type mice ($t_7=10.77$, $p<0.001$), non-stressed *Fkbp5*-knockout mice ($t_7=10.91$, $p<0.001$), and stressed *Fkbp5*-knockout mice ($t_{12}=5.20$, $p<0.001$). Nevertheless, stressed wild-type mice failed to show exploration bias toward the novel object ($t_7=0.98$, $p=0.36$), which suggests recognition memory impairments. A two-way ANOVA of the preference (stress, $F_{1,32}=27.63$, $p<0.001$; genotype, $F_{1,32}=12.85$, $p<0.01$; interaction, $F_{1,32}=2.76$, $p=0.11$), an index of recognition memory, and post hoc analyses revealed that the preference percentage of stressed wild-type mice was lower than that of the other groups (Fig. 2B).

Baseline levels of GR and pGR S211 in the wild-type and Fkbp5 knockout mice

The absence of Fkbp5 in the hippocampus of the *Fkbp5*-knockout mice was confirmed (Fig. 1). We measured GR and pGR S211 levels in the total hippocampal extracts of non-stressed wild-type and *Fkbp5*-knockout mice (Fig. 3). No differences were observed between the two groups (Fig. 3A, B; $t_6=-1.73$, $p=0.14$). However, the pGR S211 levels in *Fkbp5*-knockout mice were significantly lower than those in wild-type mice (Fig. 3A, C, D; pGR: $t_6=3.73$, $p<0.05$; pGR/GR: $t_6=3.24$, $p<0.05$). We also performed immunohisto-

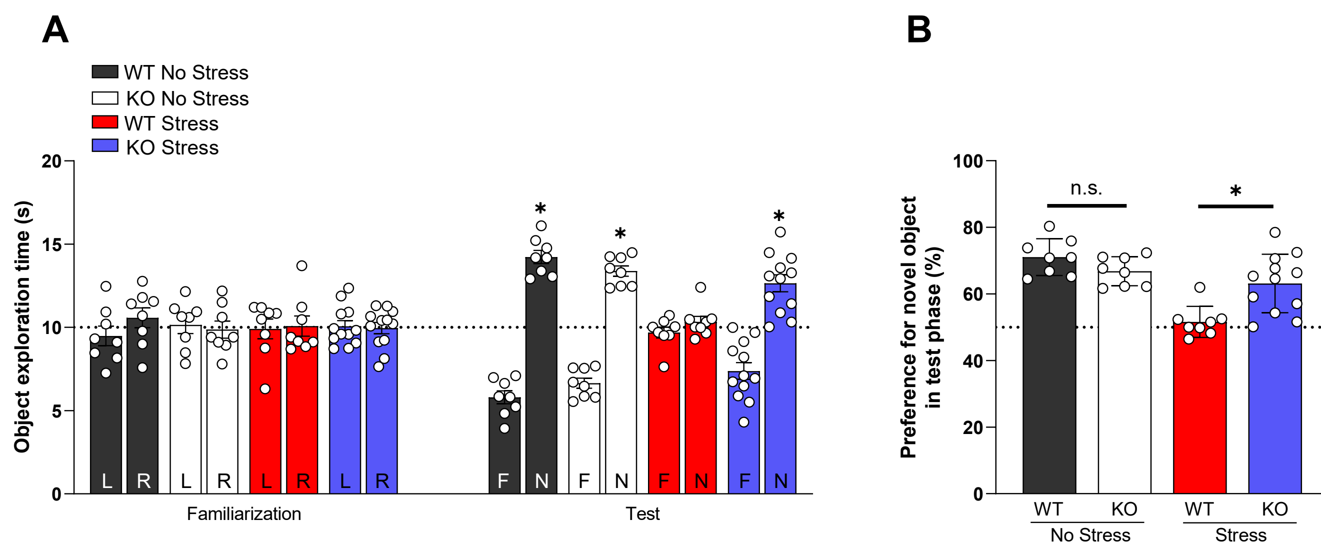
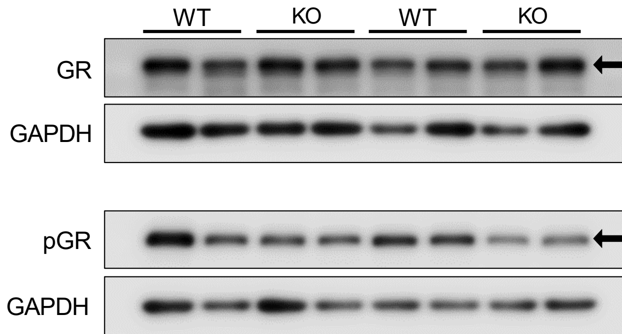
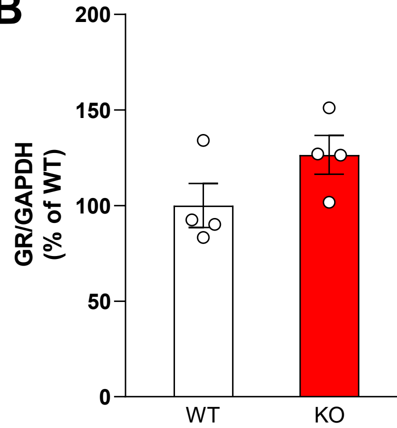
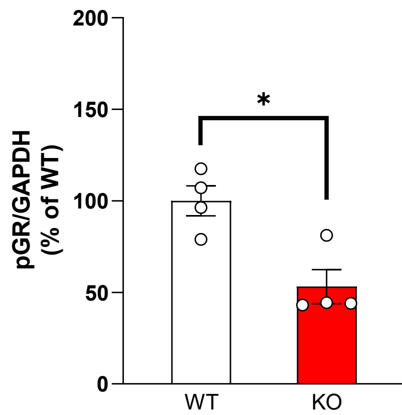
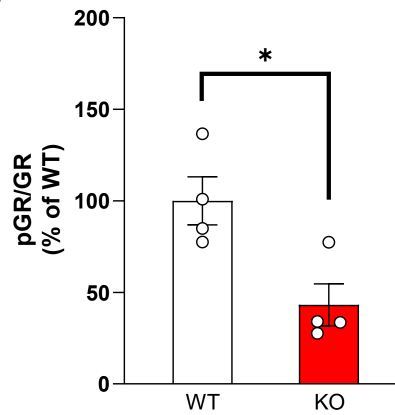


Fig. 2. Performance of wild-type (WT) and *Fkbp5*-knockout (KO) mice in the novel object recognition task. (A) A novel object recognition task with a 3-hour delay between the familiarization and test phases was conducted one hour after uncontrollable stress induction. Time spent exploring two identical objects during the familiarization phase (left) and 3 h later, one previously explored object (F) and one novel object (N) during the test phase (right). *, exploring the novel object significantly more ($p<0.05$). (B) Preference for the novel object during the test phase (30-second exploration time). ns, not significant (between-group difference in non-stressed conditions); *, The KO mice exhibited a higher preference for a novel object than the WT mice ($p<0.05$). WT no stress (n=8), WT stress (n=8), KO no stress (n=8), KO stress (n=12).









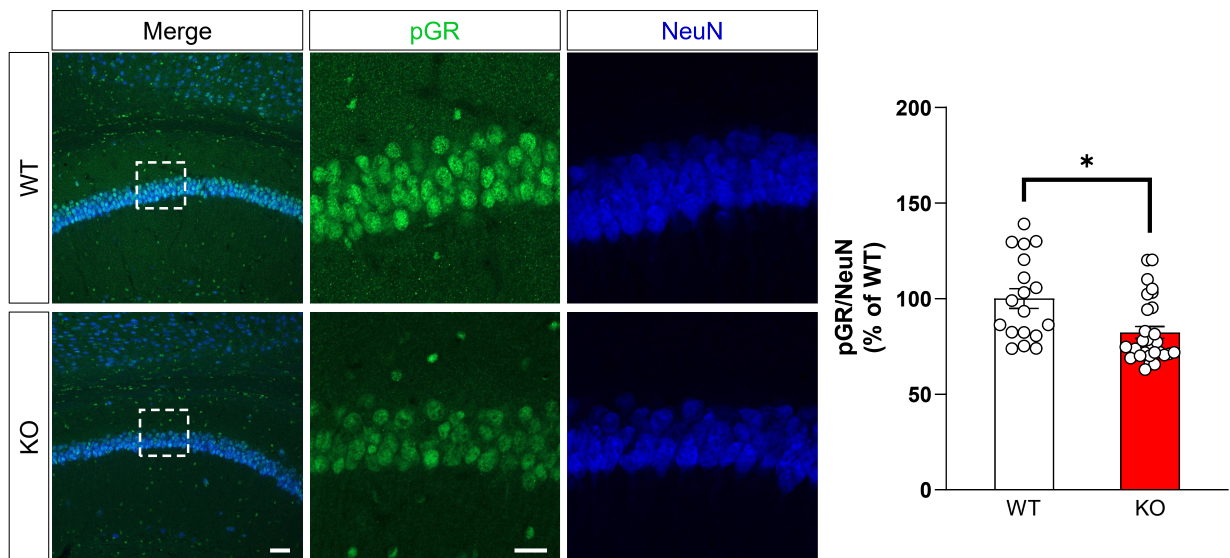


Fig. 3. Total GR and pGR S211 levels in non-stressed wild-type (WT) and *Fkbp5*-knockout (KO) mice. (A) Representative images of western blotting. (B) No differences in the total glucocorticoid receptor (GR) levels in the hippocampus (n=4 per group) were observed between groups (C, D). Hippocampal phosphorylated GR at S211 (pGR) levels were lower in KO mice than in WT mice. (E) Images showing pGR-positive (middle) and NeuN-positive signals (right) in the hippocampus of WT and KO mice. Hippocampal pGR-positive signals were lower in KO mice (n=29) than in WT mice (n=18). Scale bar: 50 μm (bottom left) and 20 μm (bottom middle). *, p<0.05. Arrows indicate the band to be quantified.

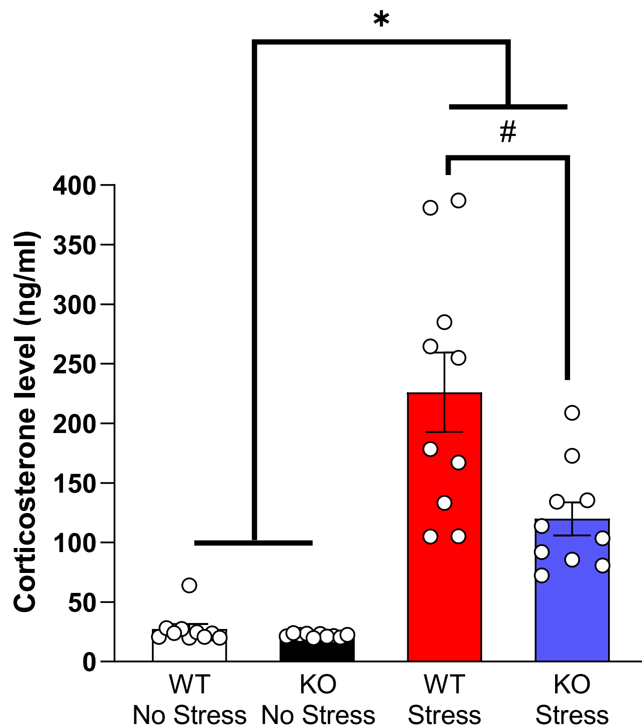


Fig. 4. Corticosterone levels in wild-type and *Fkbp5*-knockout mice. Corticosterone levels in wild-type (WT) and *Fkbp5*-knockout (KO) mice increased following stress (*, $p<0.05$). However, the stress-induced increase was much higher in WT mice than in KO mice (#, $p<0.05$). n=10 per group.

chemistry to confirm the reduction in pGR S211-positive signals (4~6 sections per mouse). The hippocampal pGR S211 intensities in *Fkbp5*-knockout mice were significantly lower than those in wild-type mice (Fig. 3E; $t_{45}=3.16$, $p<0.01$).

Lower corticosterone levels of Fkbp5-knockout mice following uncontrollable stress

A two-way ANOVA of the corticosterone levels (genotype, $F_{1,36}=9.49$, $p<0.01$; stress $F_{1,36}=67.06$, $p<0.001$; interaction, $F_{1,36}=7.74$, $p<0.01$) and post hoc analyses revealed that the corticosterone levels of stressed *Fkbp5*-knockout mice were significantly lower than those of stressed wild-type mice. However, the corticosterone levels in non-stressed wild-type and *Fkbp5*-knockout mice were low and did not differ between the two (Fig. 4).

Uncontrollable stress-induced GR translocation and pGR S211 alterations in the hippocampi of wild-type and Fkbp5-knockout mice

Representative western blots of GR and pGR S211 in cytosolic and nuclear fractions are shown in Fig. 5. We assessed stress-induced GR translocation in the hippocampi of wild-type and *Fkbp5*-knockout mice. A two-way ANOVA of cytosolic GR levels (genotype, $F_{1,40}=1.12$, $p=0.30$; stress, $F_{1,40}=0.27$, $p=0.60$; interaction, $F_{1,40}=0.41$, $p=0.53$) did not reveal any significant main factors or interaction effects (Fig. 5A, B). In contrast, a two-way ANOVA of nuclear GR levels (genotype, $F_{1,40}=0.66$, $p=0.42$; stress, $F_{1,40}=12.00$, $p<0.05$; interaction, $F_{1,40}=1.86$, $p=0.18$) revealed that nuclear GR levels were increased in the hippocampus of wild-type and *Fkbp5*-knockout mice in response to stress, indicating the occurrence of GR translocation (Fig. 5C).

In addition, we measured pGR S211 levels in the cytosolic and nuclear fractions of the non-stressed and stressed hippocampi. A two-way ANOVA of cytosolic pGR S211 levels (genotype, $F_{1,40}=3.35$, $p<0.05$; stress, $F_{1,40}=7.89$, $p<0.001$; interaction, $F_{1,40}=3.80$, $p<0.05$) and post hoc analyses revealed that cytosolic pGR S211 levels in stressed wild-type mice were higher than those in other groups (Fig. 5E). A two-way ANOVA of nuclear pGR S211 levels (genotype, $F_{1,40}=0.02$, $p=0.90$; stress, $F_{1,40}=89.98$, $p<0.001$; interaction, $F_{1,40}=2.34$, $p=0.13$) revealed that nuclear pGR S211 levels increased in the hippocampus of wild-type and *Fkbp5*-knockout mice in response to stress.

DISCUSSION

Fkbp5-knockout mice exhibit resilience to chronic restraint and social ability to overcome stress [18, 25]. Moreover, these mice exhibited enhanced stress-coping behavior and reduced hypothalamic-pituitary-adrenal (HPA) axis reactivity following exposure to an acute stressor [13, 26]. In line with the above reports, we provide evidence that Fkbp5 deficiency is one of the factors linked to stress resilience. Specifically, wild-type mice exhibited memory impairment following exposure to acute uncontrollable stress, whereas *Fkbp5*-knockout mice did not. In addition, acute stress-induced alterations in GR signaling occurred in *Fkbp5*-knockout mice, evidenced by lower corticosterone levels and less hippocampal cytosolic GR phosphorylation following acute uncontrollable stress.

The hippocampus, which is part of a system necessary for memory formation [27], is enriched with GR and terminates the stress response via glucocorticoid-mediated negative feedback of the HPA axis [4, 28]. Therefore, memory formation is susceptible to stress [2]. Stress impairs hippocampal-dependent spatial memory in rodents [1, 3, 7]. Furthermore, recognition memory has been shown to be impaired in rats with hippocampal damage and stressed rats [8, 9, 21], similar to the stressed wild-type mice used in our experiment. However, recognition memory was intact in the *Fkbp5*-knockout mice after exposure to stress. These results suggested that Fkbp5 is involved in stress resilience.

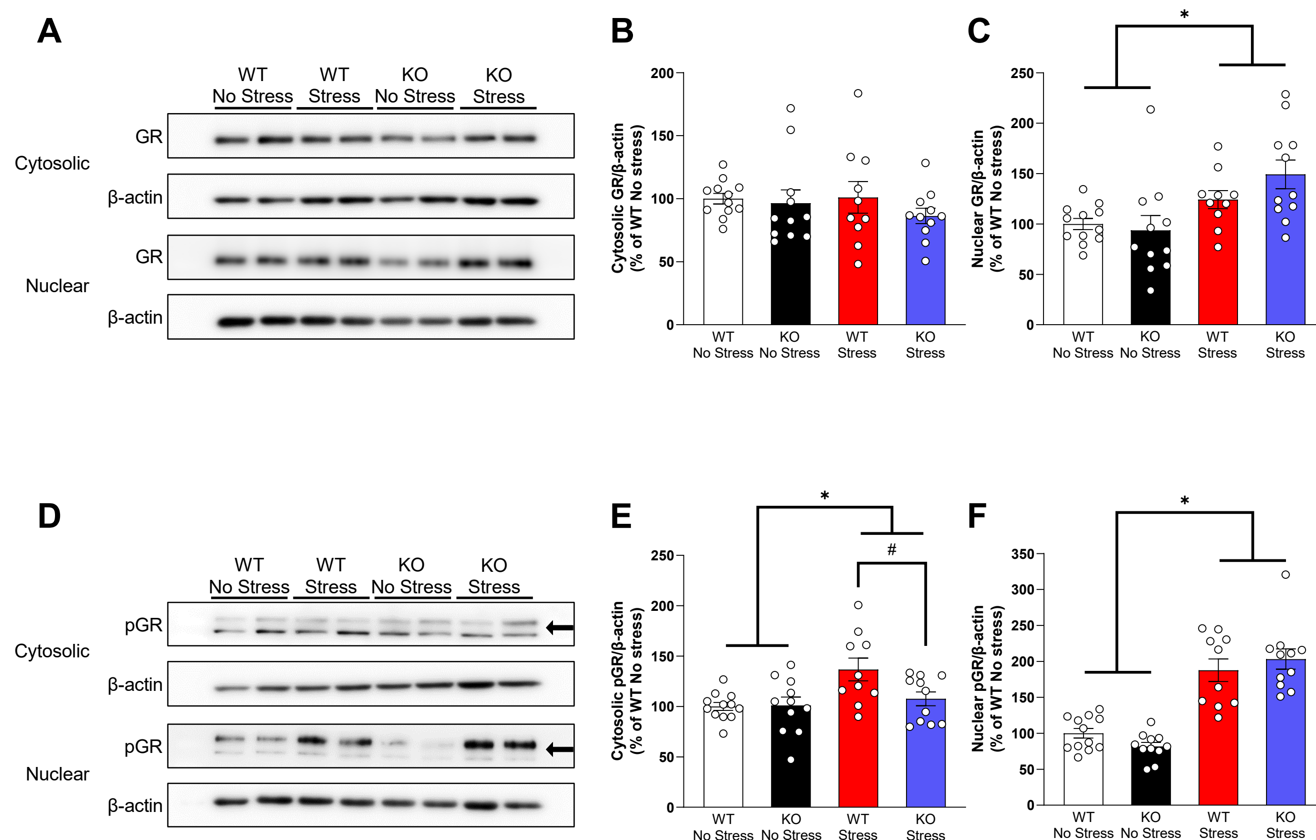


Fig. 5. Hippocampal cytosolic and nuclear expression of GR and pGR in wild-type and *Fkbp5*-knockout mice. (A) Western blot images of cytosolic and nuclear glucocorticoid receptors (GR) in wild-type (WT) and *Fkbp5*-knockout (KO) mice. (B) No differences in cytosolic GR levels between the groups were observed. (C) WT and KO mice following stress exhibited increased levels of nuclear GR. (D) Western blot images of cytosolic and nuclear phosphorylated GR at S211 (pGR) in WT and KO mice. (E) Cytosolic pGR levels were significantly increased following stress in the WT and KO mice (*, p<0.05) , but stress-induced increases in cytosolic pGR levels were observed in WT mice but not in KO mice (#, p<0.05). (F) Nuclear pGR levels were significantly increased after stress in the WT and KO mice (*), WT no stress (n=12), WT stress (n=10), KO no stress (n=11), KO stress (n=11). Arrows indicate the bands to be quantified.

FKBP5, an inhibitor of GR activity, determines the binding affinity of GR to glucocorticoids, and thus regulates the negative feedback sensitivity of the HPA axis to stress [29, 30]. Moreover, stress-induced translocation of GR into the nucleus activates an intracellular feedback loop by enhancing *Fkbp5* transcription, consequently inhibiting GR activity [16, 31]. In basal, non-stress conditions, *Fkbp5*-knockout mice exhibited no differences in corticosterone and hippocampal GR levels but had lower total pGR S211 levels than wild-type mice. In contrast, under stressed conditions, the corticosterone and cytosolic pGR S211 levels of *Fkbp5*-knockout mice were lower than those of wild-type mice. Notably, no differences between the two were observed in the total nuclear GR and nuclear pGR S211 levels under stress conditions. Therefore, these results suggest that FKBP5 mediates stress resilience by regulating GR activity. However, we measured corticosterone levels, GR translocation, and pGR S211 levels before and immediately after stress. Hence, further experiments measuring them at various time points after acute stress and dexamethasone suppression tests are required to conclude that FKBP5 deficiency increases resilience to acute stress by reduced GR sensitivity. For example, the cytosolic pGR S211 level of *Fkbp5*-knockout mice was low immediately after acute stress but will differ after one hour or more. On the contrary, GR translocation and nuclear pGR levels of *Fkbp5*-knockout mice were not different from those of the control mice immediately after acute stress but were lower relative to the control mice after one hour or more.

In the present experiment, we measured one GR phosphorylated level in the hippocampus. However, further study measuring phosphorylated GR levels at different sites is needed to corroborate the findings. The GR is phosphorylated at three major sites (S203, S211, and S226) on the N-terminal side, and an interaction between multiple phosphorylation sites is reported to be essential for GR activation and repression. For example, the phosphorylated GR level at S203 was higher when no GR phosphorylation oc-

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curred at S226 and vice versa, indicative of intersite dependency [32]. Moreover, GR transcriptional activation increases when S211 phosphorylation exceeds S226 [20].

Most studies using *Fkbp5*-knockout mice by replacing LacZ and neo cassettes in the exon 2, including ours, have reported behavioral phenotypes indicating stress resilience [13, 18, 25, 26, 33]. In the forced swim test, these *Fkbp5*-knockout mice showed reduced immobility [33]. However, *Fkbp5*-knockout mice generated by inserting a beta-geo-trapped cassette between the exon 4 and exon 5 regions exhibited increased immobility in the forced swim test and reduced saccharin intake [34]. Notably, FKBP5 overexpression impairs cognitive function [35]. Further studies to reveal changes in signaling (e.g., GR signaling) in a brain structure known to mediate memory and stress in a genetically engineered *Fkbp5* mouse model would explain these behavioral discrepancies.

Polymorphisms in *Fkbp5* have been linked to stress-related psychiatric disorders [16, 36, 37]. *Fkbp5* gene variants in patients with posttraumatic stress disorder exhibit altered sensitivity of the GR. Pharmacological inhibition of *Fkbp5* improved stress resilience [17, 19, 38]. Therefore, the GR-FKBP5 protein complex may be a therapeutic target and diagnostic marker for stress-related psychiatric disorders.

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DECLARATION OF COMPETING INTEREST

The authors declare no conflicts of interest.

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