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스트레스 유발 마우스모델에서 뇌염증 및 신경행동 장애 변화

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Neuro-inflammation induced by restraint stress causes impairs neurobehavior in mice

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ABSTRACT

Background : Behavioral stress has been suggested as one of the significant factors that is able to disrupt physiological systems and cause depression as well as changes in various body systems. The stressful events can alter cognition, learning, memory and emotional responses, resulting in mental disorders such as depression and anxiety.

Results : We used a restraint stress model to evaluate the alteration of behavior and stress-related blood parameter. The animals were randomly divided into two groups of five animals each group. Furthermore, we assessed the change of body weight to evaluate the locomotor activity as well as status of emotional and anxiety in mice. After 7 days of restraint stress, the body weight had significantly decreased in the restraint stress group compared with the control group. We also observed stress-associated behavioral alterations, as there was a significant decrease in open field and forced swim test, whereas the immobilization time was significantly increased in the stress group compared to the control group. We observed the morphological

changes of neuronal death and microglia by immunohistochemistry and western blot. In our study restraint stress did not cause change in neuronal cell density in the frontal cortex and CA1 hippocampus region, but there was a trend for an increased COX-2 and iNOS protein expression and microglia (CD11b) in brain, which is restraint stress.

Conclusion : Our study, there were significant alterations observed in the behavioral studies. We found that mice undergoing restraint stress changed behavior, confirming the increased expression of inflammatory factors in the brain.

Key words : restraint stress, anxiety, depression, neuro-inflammatory, neurobehavior

Introduction

Stressors can disturb physiological/psychological homeostasis as well as disturb any already known conditions, including affecting the function and morphology of the hippocampus, as defined in biological systems[1]. Stress mediates an inhibitory effect, although the exact cellular mechanism is not known. Stress-induced anxiety suppresses behavior in different animal models[2,3]. Stress can be limited to an organism's homeostatic balance and induce physical and mental strain[4]. Restraint stress is a popular and expedient method to induce both psychological and physical stress resulting in defined mobility and aggression. Restraint is a preferred means of stressing animals, largely because it is a straightforward and painless method[5]. An immobilized animal has been extensively used as a restraint stress model for the study of biological, biochemical and physiological responses related to stress[6]. It has been assumed that the integration of stress signals that occur in different regions of the body also occur in the hypothalamus[7]. There is evidence that the sympathetic nervous system has an important response to the stress induced model. One study has shown that increasing restraint stress duration can increase global activation of the sympathetic nervous system[8]. Complex interactions between the hypothalamic-pituitary adrenal axis (HPA) and various stress inputs with components of the embedded system determine the outcome of the stress response[9]. Elevated levels of plasma corticosterone and adrenocorticotropic hormone (ACTH) have been reported to be associated with immobilization-induced stress[10].

Decreased physical activity in adolescents and young adults can lead to an incidence of anxiety disorders as well as other circulation mental illnesses[11]. The glucocorticoids released from the adrenal cortex are partially modulated by the hypothalamus and the anterior pituitary, which secrete stimulating hormones such as corticotrophin releasing hormone and adrenocorticotropic hormone, respectively[12]. When there is an energetic need, there is a decrease in the production of stimulating hormones when energetic needs are comprehended as stable[13]. At resting or non-stress conditions, the baseline plasma of glucocorticoid levels vary predictably across a 24 hours period in some

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species[14].

Many of these effects are mediated by stress-associated neurochemicals and hormones that correspond to oxidative stress[14]. Sub-acute immobilization stress increases the infarct volume in the brain and acts as an excitotoxicity and brain inflammatory mechanism, which exacerbates neurological outcomes[15]. Restraint stress increases brain iNOS activity and expression 6 hours after stress incidence, and long-time stress produces a high level of iNOS expression and activity[16]. iNOS caused by the harmful effects of long-term stress-induced damage to the mitochondria are destroyed and the accumulation of blood-brain barrier membrane of the aldehyde product (peroxide product)[17]. The mechanisms of stress (acute and chronic)-induced increase in iNOS activity, NFκB blockade (by preventing stress-induced IkBα decrease) and inhibition of TNF-α release in stressed animals[18]. The subacute restraint stress model can assess the potential therapeutic efficacy of the disease in a variety of behavioral models, which can be useful in studying stress and pain, emotional anxiety states and connection mechanisms[19]. Previous studies have shown that chronic and acute restraint stress procedures alter certain behavioral parameters in mice. In this study, we attempted to investigate the restrainer stress model and associated effects on neurogenesis and neurobehavior in mice.

Materials and Methods

Animals

Male ICR mice (6 weeks old) were purchased from Samtako, Inc. (Osan, Korea). They were housed under standard conditions (23 ± 1°C, 50 ± 5% humidity), with 5-6 animals per cage and a 12-h light/dark cycle. Food and water were available ad libitum. The animals were handled in accordance with the guidelines established by the Institutional Animal Care and Use Committee of KIOM (Daegu, Korea), under reference number KIOM- D-16-015. The experiments were performed according to the guidelines of the Animal Care and Use Committee at KIOM.

Restraint stress procedure

Mice were randomly divided into the following two groups: the restraint stress group and the control group. Restraint stress was induced by placing mice in ventilated 50 mL conical tubes without penning their tail for 1 week, which was a close fit for the mice, as described previously (2 h/day between 10 am and 12 pm, 7 days a week)[20]. Control stressed animals were anaesthetized, but they were not placed in tubes and were replaced in their home cages.

Behavioral Testing

Open field. Open field tests were performed as previously described[21]. Video tracking was analyzed using the SMART V3.0 video tracking system (Panlab, Barcelona, Spain). During the 30-min trials, the distance moved and time spent in the arena by the mice was recorded under dim lighting, along with a delineated 'periphery zone' and a delineated 'center zone' (30 cm×30 cm×25 cm) (Figure 2a).

Forced swim test. Forced swim tests were performed as previously described[21]. Mice were videotaped using the SMART V3.0 video tracking system (Panlab, Barcelona, Spain) while swimming in an acrylic Pyrex glass beaker (10 cm × 25 cm) containing 24±1 for water for 5 min. The water in the beaker was changed after each trial. Two trained and blinded observers scored the videotape manually. The total immobility was measured as the time spent without any motion except for single limb paddling to maintain flotation. The latency to immobility was assessed as the time elapsed until the mouse first becomes immobile.Sucrose preference was performed as previously described[21].

Plasma analysis

A Hitachi 7080 (Tokyo, Japan) biochemical analyzer was used to measure the serum concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin (ALB), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein-cholesterol (LDL-C), triglyceride (TG), creatinine(CRE), total protein (TP), lactate dehydrogenase (LDH) as previously described.

Corticosterone and cortisol level measurement

Corticosterone and cortisol levels were measured with an enzymatic immuno-assay kit (R&D Systems, Ins. Ann Arbor, MI, USA) following the manufacturer's instructions with modifications. Briefly, mice were decapitated, and 1 mL of blood was collected and mixed with 30 μL of 100 mM EDTA immediately after the final restraint stress. Samples were centrifuged (15 min, 1000 ×*g*, 4°C). Each supernatant was added to a 96-well plate at two dilutions (1:300) in duplicate and subjected to the immunoassay. The optical density of the enzyme products was read at 405 nm.

Staining for Immunohistochemistry

Immunohistochemical staining was used to study processes of glia activation and neuron viability. Antibodies NeuN (neuron nuclear marker protein) and mouse anti-mouse CD11b (OX-42) were used. Series of 10-μm-thick sections were cut from paraffin embedded specimens. After deparaffinization and endogenous peroxidase activity was blocked by 5min incubation in 3.0% H_2O_2 to block non-specific endogenous peroxidase activity. After washing in PBST, the sections were reacted with mouse anti-neuronal nuclei (NeuN) monoclonal antibody (1:100, Millipore, Bedford, MA, USA) and mouse anti-mouse CD11b (1:50, BD PharMingen, San Diego, CA, USA) for overnight at 4℃, and consequently with biotinylated universal anti-mouse, -goat, and -rabbit immunoglobulins in PBST for 15 min, 3times. After washing in PBST, the sections were incubated with streptoavidin conjugated to horseradish peroxidase (HRP) in PBST for 30 min. Finally, the sections were reacted with a solution containing diaminobenzidine (DAB) and hydrogen peroxide (0.001%). Counterstaining of sections by toluidine blue or hematoxyline was performed. Slides were dehydrated and embedded in Permount according to standard protocol. Histopathological changes were observed under 50X, 100X and 200X magnification field of the immunoreactivity for NeuN and CD11b.

Western blot

Seven days after restraint stress, the mouse were sacrificed and the brains were removed. For western blot analysis, the whole brains were homogenized at 4 °C in RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1mM PMSF, 1mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS)]. The lysates were cleared by centrifugation at 12,000 ×g for 10 min, and the total cellular protein (20 μg/lane cytosolic fractions) was separated on a 12% SDS-polyacrylamide gel and electro-transferred onto PVDF membranes (Hybond ECL, Amersham International, Bucks, UK). Blots were rinsed with 1×Tris-buffered saline and 0.1% Tween 20 (TBST), and then blocked with 5% BSA for 1 h at room temperature. The membranes were incubated overnight at 4 °C with specific primary antibodies for iNOS (1:1000, Santa Cruz Biotechnology Inc., CA, USA), COX-2 (1:500, Cell signaling), NeuN (1:500, Millipore, Bedford, MA, USA), mouse anti-rat CD11b (1:100, BD PharMingen, San Diego, CA, USA) and β-Actin (1:5000; Sigma-Aldrich) as a control. After incubation with primary antibodies, the membranes were washed 5 times for 10 min each and then incubated with secondary antibody conjugated to horseradish peroxidase (anti-rabbit, 1:2000; Santa Cruz). Immunoreactive proteins were detected by the enhanced chemiluminescence system with a BioRad

Clarity Western ECL Substrate and ChemiDoc Touch Imaging System (Bio-Rad, Hercules, CA).

Statistical analysis

Statistical analyses were performed using Sigma Stat 3.1 software (System Software, Inc., Chicago, IL, USA). For body weight gain, differences among groups were analyzed by repeated measures ANOVA. Differences among groups were analyzed by Student's t-test or one-way ANOVA, followed by Bonferroni's post hoc test.

Results

Effects of restraint stress on body weight and food intake

The study was undertaken to assess the various neurobehavioral changes in mice applying restraint stress in mice after exposure to restraint stress. The experiment was carried out with 15 healthy male mice weighing approximately 30±2 g. The animals were randomly divided into the following two groups: Group I-control group (5 mice) and Group II - restraint stress group (10 mice). The restraint stress group mice were placed in 50 mL conical centrifuge tubes with multiple punctures to allow ventilation for 2 hours per day between 9.30 am and 11.30 am for 7 days. The effects of the daily restraint stress for 7 days on body weight and food intake are shown in Fig. 1. While the body weights of the control mice gradually increased over the course of the 7-day experiment, the body weights of the stressed mice dropped sharply during the first day. As a result, the stressed mice had significantly lower body weights than the control mice during the entire experimental period (Fig. 1A). The total food intake of the stressed mice also decreased during the first few days of the experiment. The daily food intake of the stressed mice then gradually recovered substantially after 5 days of the experiment, and it remained significantly lower at nearly every time point (Fig. 1B).

Tissue weights

After the experiment, all organs were harvested and the weight of each organ was calculated. There was no significant difference in the weights of the organs. Because each group had only 5 animals, no statistics were calculated. However, the expected effects of stress on the brain and kidneys could be detected. The relative brain weight increased, and the relative kidney weight decreased after stress on day 7 (Fig. 1C).

Serum blood parameter

The levels of TC, LDL-C, HDL-C and TG of the control mice were 625.0 ± 45.4 mg/dL, 58.0 ± 4.6 mg/dL, 547.0 ± 45.9 mg/dL, and 555.0 ± 39.8 mg/dL. Compared to the control group, the stressed mice presented with a higher level of TC, 668.2 ± 27.2 mg/dL, and a higher level of TG, 640.9 ± 41.5 mg/dL, but there were no significant differences ($P>0.05$) in either parameter. A higher level of LDL-C, 72.5 ± 3.1 mmol/L, indicated a remarkable statistic difference ($P<0.05$ or $P<0.01$) (Fig. 1D). Compared with the activities of ALB, AST, ALT and LDH of the control group, which were 18.5 ± 0.6 g/dL, 250.0 ± 22.4 IU/L, 120.0 ± 9.4 IU/L and 4305.0 ±547.6 IU/L, respectively, the mice in the restrainer-induced stress model group had a lower level of ALB, 16.4 ± 0.5 g/dL; a higher level of AST, 368.2 ± 15.1 IU/L; a higher level of ALT, 154.5 ± 8.8 IU/L; and a significantly higher level of LDH, 6129.5 ± 397.9 IU/L. The differences observed in these two groups were statistically significant ($P<0.01$); however, the control group did not show any significant differences ($P>0.05$) (Fig. 1D).

Behavioral Testing

Open field.

The open field test was performed as described previously[11]. Locomotor activity was measured in an open field (30 cm×30 cm×25 cm). Mice were individually placed at the center of the open field,

and the locomotive trajectory, distance traveled, and times spent in the center and on the periphery were recorded for 30 min. Acute stress exposure resulted in depressive-like symptoms. The center area of the open field was defined as the inner rectangular area that constituted 30% of the open field. Compared to the activities of the resting and slow time of the control group (51 ± 19 and 47 ± 18, respectively), the activities of the resting and slow time of restrainer-induced stress model group were much different (17 ± 8 and 78 ± 7, respectively). There was a significant difference ($P<0.01$ or $P<0.05$) upon comparison of the two groups (Fig. 2).

Forced swim test.

Forced swim tests were performed as previously described [19]. Mice were videotaped while contained in acrylic Pyrex glass beaker of water at 25±1°C for 5 min. Water in each beaker was changed after each trial. Two trained and blinded observers scored the videotape manually. The total immobility was measured as the time spent without any motion except for single limb paddling to maintain flotation. Latency to immobility was assessed as the time elapsed until the mouse first became immobile.

To inspect how manipulation of restrainer stress model activity alters baseline depression-like behaviors, we performed the forced swim test, an established measure of behavioral 'despair'. We found a main effect of stress on time spent immobile (t-test: confidence interval 95%, $p<0.01$), with post-hoc analysis revealing a significant increase in time spent immobile in the restrainer stress group compared with the control group (Fig. 3A). However, there were differences in latency to immobility ($p<0.05$; Fig. 3B).

Serum corticosterone and cortisol level.

Physical stress resulted in restricted mobility and aggression. The physiological response to stress occurs through activation of the HPA axis and causes the release of corticosterone and cortisol. Restraint stress no changed plasma corticosterone and cortisol levels immediately after restraint termination by day 7. Mice were sacrificed at different times of day to collect blood for the control values for the cortisol and corticosterone assay. The blood corticosterone and cortisol level after the final restraint stress was no changed in control group and restrainer stress group (Fig. 4).

Effect of restraint stress on activation of neuronal cell and microglia in brain

To investigate the effect of restraint stress on the activation of inflammatory cells in brain, we observed the morphological changes of neuronal death and microglia by immunohistochemistry and western blot. Restrain stress induced loss of NeuN-positive neuronal cells (Fig. 5A,C) and activation of inflammatory cells such as CD11b-positive microglia (Fig. 5B,C) and iNOS, COX-2 (Fig. 5D) in the brain. In contrast to brain, the immunoreactivity of NeuN was almost faded in the cortex area after restrain stress, but there was little change in the hippocampus (Fig. 5A). Large amount of CD11b-immunoreactive cells in the cortex and hippocampus were remained after restrain stress (Figs. 5B). These inflammatory cells were maintained at resting morphology by restrain stress group similar to the control group. As shown in Figure 5 C and D, the expression of CD11b, iNOS and COX-2 was significantly increased in restraint stress-induced brains compared with control group ($P<0.01$, respectively). And decreased the expression of NeuN-neuronal cell (Fig. 5D) compared with the control group.

Discussion

In this study, we investigated the behavioral disturbances and inhibition of behaviors in a restraint-induced stress mouse model. The distinct advantage of using immobilization as a stressor lies in the fact that it creates both physical and

psychological stress. Stress causes a significant reduction in motor activity and motivation in the animal[22]. Experiments using rodent models to investigate behavioral changes caused by chronic pain have reported somewhat inconsistent results. In one study, behavioral changes, such as anxiety, appear 4 weeks after stress, and depression occurs 6−8 weeks after stress in the neuropathic animal model[23]. Stress has been proven as part of the mechanisms associated with anxiety and depression. In other studies, differences in experimental time in painful animal models found no change in behavior[24], only an increase in behaviors such as anxiety[25], depression−like behaviors[26], or behavior−like depression with cognitive deficiency[27]. The subacute restraint stress model can be useful in studying stress and pain, emotional anxiety states and connection mechanisms that evaluate the potential therapeutic efficacy of disease in various behavioral models. There are few studies on subacute restraint stress in mice in the study of neural behavioral parameters.

We found that acute restraint stress affects mouse behavior, particularly in the open field test and forced swim test. The relaxation and slow time of the field score in the open field test reflects the excitement of the mouse in the environment[28]. The activity of the restrained group is higher than that of the control group, and as a result, stress increased excitatory activity of the mouse. We also compared these groups using the forced swim test, which was a response of the mouse's survival mechanism, whereby active swimming is used as a measure of behavior such as helplessness or depression during the test period. Immobility increased in the restraint groups, in accordance with increased susceptibility to behavior such as depression.

Additionally, we investigated the effects of restraint stress on body weight and food intake in mice. Restraint stress rapidly induced a marked decrease in body weight that may be due to a reduction of food intake. However, there was a recovery to 90% of food intake compared to the control, which was caused by stress during the exposure period and did not match the number of times the equivalent weight. Although stress−induced degradation of body weight can be attributed initially to decreased food intake, weight loss can be due to increasing energy consumption and body temperature[29]. In particular, previous reports have shown that rats exposed to chronic restraint did not recover after the elimination of stress and showed rapid weight loss[30]. In addition, stress may modify pathways that normally respond to a reduction in body weight. It has been reported that stress−related pathways act against a mechanism that normally promotes recovery of weight to normal levels[31]. This reaction leads to an increase in blood glucose levels and depressed immune functions. In addition, stress has been shown to play an important role in the development of cardiovascular disease[32−34] and can inhibit the metabolism of lipids and the metabolism of glycols[35]. Another study has been reported that stress may have some effect on the liver and oxidative stress in brain[36−39].

Although a considerable amount of evidence has shown that physical and psychological stress elevates plasma levels of several cytokines. In our study restraint stress did not cause change in neuronal cell density in the frontal cortex and in the CA1 region of hippocampus, while a trend for an increased COX−2 and iNOS protein expression and microglia (CD11b) was observed in cortex and hippocampus, which is particularly sensitive to stress. The observed increased expression and activity of inflammatory in brain cortex after a 4−6h exposure restraint stress in rat[17]. NO may also be involved in the pathophysiology of anxiety / depression because it interferes with the various components and underlying mechanisms of the stress response.[40]. High expression isoforms of iNOS (NOS−2) are associated with cytotoxicity in

many cell systems, including the brain. In this context, it was demonstrated that the stress to induce the expression of iNOS in the brain of the mouse, and its inhibition prevents the cellular damage caused by stress in this model. On the other hand, the COX pathway has also been implicated in stress-induced brain damage. COX-2 is derived from stress and is involved in the damage associated with this condition[41]. In our study, Stress-induced microglia activation and macrophage recruitment to the brain contributes to development of prolonged anxiety-like behavior (Fig.5). Similar to iNOS, depending on the status of the inducible enzyme, the immediate gene COX-2 promoter is dependent on NF-κB activation in stress[42]. Chronic restraint stress is associated with increased oxidative stress, which has been demonstrated by elevated levels of lipid peroxidation and a deficiency of endogenous enzyme antioxidants. It has been shown that the nervous system is highly sensitive to oxidizable substrates and high oxygen tension. Immobilization increased lipid peroxidation in the liver[38], suggesting that this tissue may be compromised in an acute stress model. As shown in our results, restrainer stress caused significant increase in serum ALT, AST compared to the control group. However, there was no significant effect on the stress hormone (cortisol and corticosterone). Increased oxidative stress by restraint stresses increases the risk of many diseases due to oxidative damage of DNA, proteins and lipids catalyzed by reactive oxygen species[43-45]. Furthermore, acute stress increases the decomposition of hepatic glycogen without the special characteristics of stress, release of hepatic cord hepatocytes or without hyperglycemia[46] and reduced triglyceride levels, the most stored lipid in the liver[47]. Our results indicate that restraint and confrontation stress induce a roughly similar increase in the plasma TC, TG, LDL-C and LDH concentration. Recent animal studies have shown that obesity affects the expression of hormones leptin, lipids, and the oxidation in the liver, which are mainly expressed in the coding and adipocytes by the genes.

In summary, restraint stress produces inevitable physical and mental stress, and a significant behavioral changed can be observed. Thus, early weight loss is caused by a reduction in daily food intake in the induced- restraint stress animal model; however, in the present study, restraint stress-induced mice displayed a decrease in body weight without a reduction in food intake. In addition, the stress procedure affected the changes in the locomotor activity and changed behaviors that are related to emotion and anxiety in behavior test. Stress often indicates anxiety / depression of two behavioral changes. Inflammatory mediators are implicated in neurobiological of nerve cell death and neuro-inflammation changes that promote restraint stress-induced behavior.

Authors' contributions

KI Park conceived and designed the study, and supported all materials. TW Oh, HJ Do, YW Kim, BW Lee and KY Kim performed the experiments, statistical analysis. TW Oh wrote the manuscript. KI Park and JY Ma reviewed the literature, revised the manuscript and coordinated the study. All authors read and approved the final version of the manuscript.

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Availability of data and materials

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Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable

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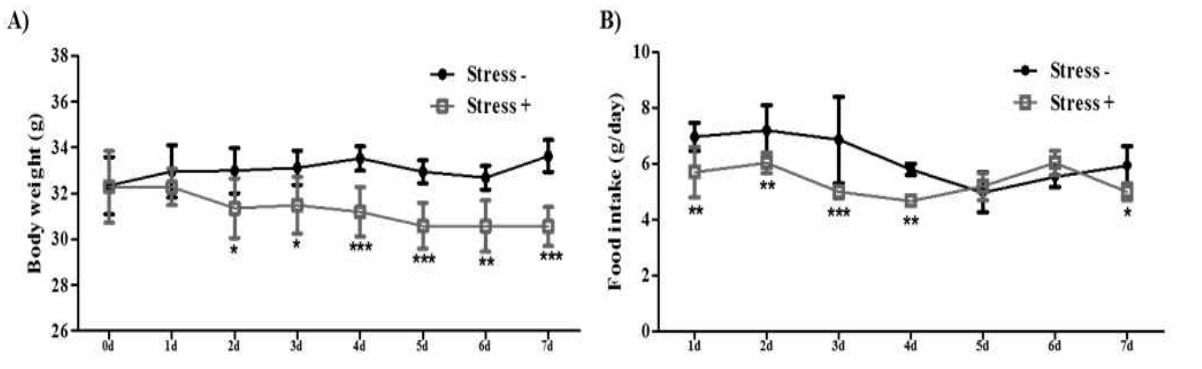
Figure legends



C)

Treatment	Liver weight (g)	Liver weight (% body weight)	Kidney weight(g)	Kidney weight (% body weight)	Spleen weight(g)	Spleen weight (% body weight)	Brain weight(g)	Brain weight (% body weight)
Stress -	1.87 ± 0.06	5.57 ± 0.21	0.54 ± 0.02	1.59 ± 0.06	0.11 ± 0.01	0.34 ± 0.02	0.49 ± 0.01	1.45 ± 0.02
Stress +	1.59 ± 0.03	5.23 ± 0.10	0.45 ± 0.01	1.48 ± 0.05	0.12 ± 0.02	0.38 ± 0.07	0.46 ± 0.01	1.53 ± 0.03

D)

Treatment	AST IU/L	ALT IU/L	ALP IU/L	ALB g/dL	TC mg/dL	LDL mg/dL	HDL mg/dL	TG mg/dL	CREA mg/dL	UREA mg/dL	TP g/dL	LDH IU/L
Stress -	250.0 ± 22.4	120.0 ± 9.4	390.0 ± 12.7	18.5 ± 0.6	625.0 ± 45.4	58.0 ± 4.6	547.0 ± 45.9	555.0 ± 39.8	2.5 ± 0.0	124.5 ± 5.0	27.0 ± 0.5	4305.0 ± 547.6
Stress +	368.2 ± 15.1***	154.5 ± 8.8*	320.5 ± 28.0	18.4 ± 0.5	668.2 ± 27.2	72.5 ± 3.1*	560.0 ± 19.5	640.9 ± 41.5	2.5 ± 0.0	100.2 ± 5.6*	26.1 ± 0.6	6129.5 ± 397.9*

Figure 1. Changes in the body weight, food intake, tissue weight and serological markers in the restraint-induced stress model.
(A) Changes in body weight in the restraint-induced stress model. (B) Changes in food intake in restraint-induced stress model. (C) Changes in tissue weight in restraint-induced stress model. (D) Changes in the serological markers in restraint-induced stress model. Histogram values are presented as the means±SD of three independent experiments. *p<.05 **p<.001 and ***p<.0001 versus non-treated Stress- group by unpaired 2-tailed t-test.

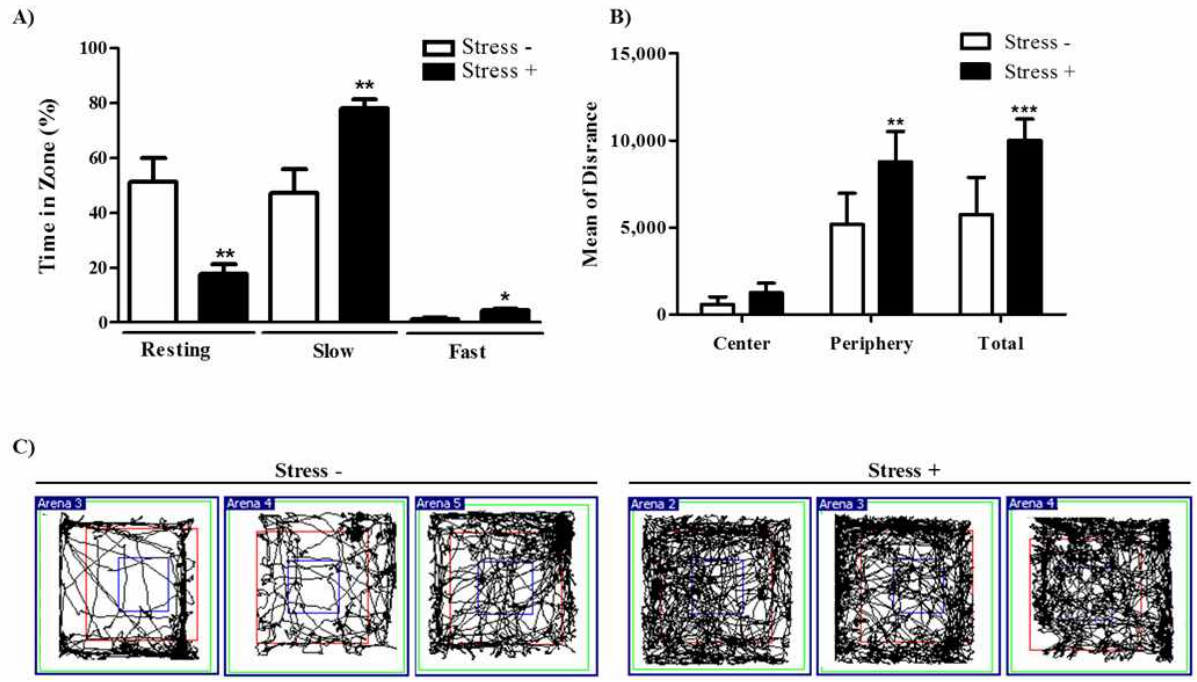


Figure 2. Neurobehavioral changes observed in the open field tests (OFTs) in restraint-induced stress model.

(A) Change of resting and movement (slow and fast) time in acute-stress mice. (B) Distance travelled in center and periphery zone by mice. (C) Representative traces of mouse movement during OFT. Values are expressed as the means±SD of three independent experiments. *p<.05 **p<.001 and ***p<.0001 versus non-treated stress group by unpaired 2-tailed t-test. n = 5 mice per group.

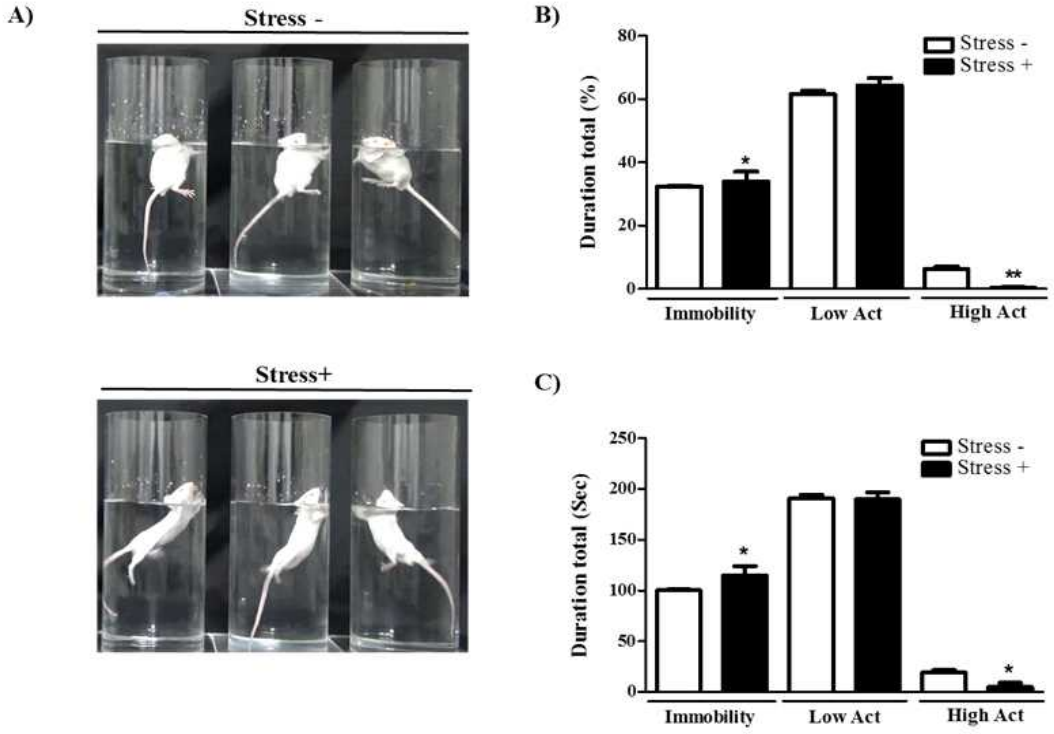


Figure 3. Forced swim test (FST) in the restraint-induced stress model.
(A) Representative video track software in forced swim test (B) Immobility duration in the FST. (C) Total duration moved during a 5-min period of time (sec). Histogram values are presented as the means±SD of three independent experiments. *p<0.05 and **p<0.01 versus non-treated Stress- group by unpaired 2-tailed t-test.; n = 5 mice per group.

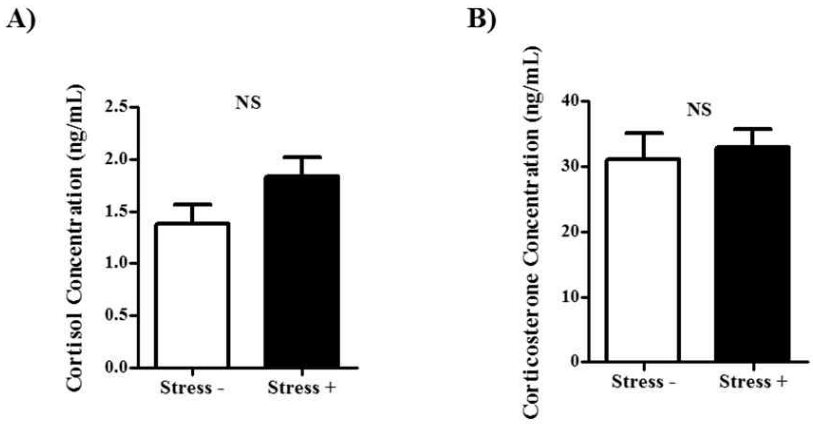


Figure 4. Cortisol and corticosterone levels in sera collected from control and restraint-induced stress groups.
(A) Cortisol and (B) corticosterone levels were measured 1 week after restraint-induced stress was initiated. Values are expressed the means±SD of three independent experiments.

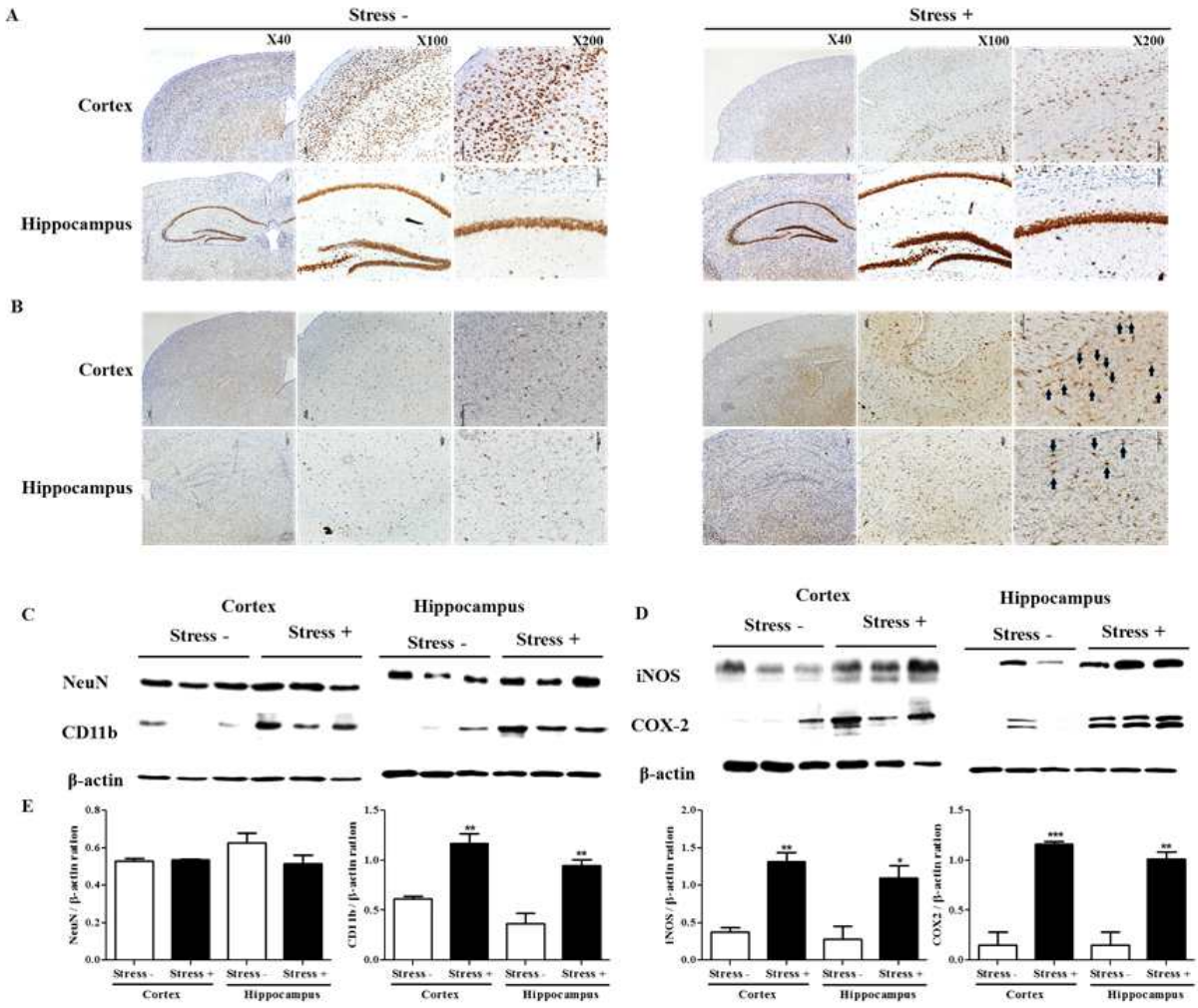


Figure 5. Neuronal cell and microglia expression in brain from control and restraint-induced stress groups.

After restrain stress, brain tissues were immune-stained by anti-NeuN (A), anti-CD11b (B) antibodies. The morphological changes of neuronal cells (A), and microglia (B) were observed in the penumbra of ischemic mice by microscope (original magnification ×40, ×100 and ×200). The photograph is a representative image of three different tissues. And the protein was isolated from brain tissues, and detected the expression of NeuN, CD11b (C), iNOS, COX-2 (D) and NeuN, CD11b, iNOS, COX2 (E) were calculated by normalization to β-actin. Data in the histogram are expressed as means±SD of three independent experiments (n = 3 per group). **P <0.01 vs. stress (−) vs. stress(+).