

Original Article

## Application of Single Prolonged Stress Induces Post-traumatic Stress Disorder-like Characteristics in Mice

Ken-ichi Tanaka<sup>a,b</sup>, Takao Yagi<sup>b,c</sup>, Takeshi Nanba<sup>b,c</sup>, and Masato Asanuma<sup>c,d</sup>

<sup>a</sup>Physiology and Pharmacology, School of Health and Social Services, Saitama Prefectural University, Koshigaya, Saitama 343-8540, Japan, <sup>b</sup>Department of Clinical Pharmacy, Shujitsu University School of Pharmacy, Okayama 703-8516, Japan, <sup>c</sup>Departments of <sup>c</sup>Brain Science, <sup>d</sup>Medical Neurobiology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan

We tried to clarify the applicability of a single prolonged stress (SPS) protocol as post-traumatic stress disorder (PTSD) model in mice. To investigate PTSD pathophysiology, we conducted hypothalamo-pituitary-adrenal (HPA) negative feedback testing at 1, 4, 8 and 12 weeks after the SPS by administrating a dexamethasone (DEX) suppression test. The SPS induced over-suppression of the HPA system by DEX treatment at 8 and 12 weeks. To investigate PTSD-like behavioral characteristics, we subjected mice to testing in a light/dark box (to assess anxiety), a Y-maze (working memory), a cliff avoidance (visual cognition), and an open field (locomotor activity) at 1, 4, 8 and 12 weeks after the SPS. In the light/dark box test, the SPS-applied mice spent significantly less time in the light box at 8 or 12 weeks. In the cliff avoidance test, the SPS-applied mice spent significantly less time in the open area at 1 week. However, in both the Y-maze test and the open field test, SPS-applied mice tended toward slight decreases in a time-dependent manner until 12 weeks. Therefore, SPS-applied mice may thus be useful for assessing characteristics relevant to PTSD that coincide with changes in the HPA axis.

**Key words:** PTSD, single prolonged stress, corticosterone, mouse

Post-traumatic stress disorder (PTSD) is a stress-related mental disorder caused by exposure to severe traumatic events, such as war, violent personal assault, or natural disasters, and PTSD is characterized by intrusive memories (flashbacks), hyperarousal, and avoidance symptoms. The pathophysiology of PTSD is still unclear, but both fear conditioning and sensitization are thought to play crucial roles [1]. Various hypothalamo-pituitary-adrenal (HPA) axis abnormalities have also been reported in a PTSD-enhanced suppression of cortisol in response to an administration of dexamethasone (DEX) [2,3]. Generally speaking, animal models of anxiety including those of PTSD can be

grouped into two main classes: the first involves the conditioned responses of animals to stressful and often painful events such as electric foot-shock, and the second includes ethology-based protocols and involves the spontaneous or natural reactions of animals such as flight, avoidance, or freezing, to investigate stimuli that do not explicitly involve pain or discomfort [4]. Most models involve the exposure of subjects to external cues paired with either foot shock, bright light, or a predator, or internal cues such as drug-state stimuli that are assumed capable of inducing anxiety in animals [5].

In the case of PTSD, the mouse model involving repeated exposures to conditioned fear (CF) stress and the rat model involving single prolonged stress (SPS) are

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\*Corresponding author. Phone & Fax: +81-48-971-0500

E-mail: tanaka-ken-ichi@spu.ac.jp (K. Tanaka)

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the two common animal models for PTSD research [3,6]. The CF mouse model shows specific fear responses, called contextual fear and cued fear, to training environments and cues [6]. However, that model cannot mimic all of the symptoms of PTSD patients. For example, it is difficult to use the CF model to investigate the function of HPA axis and some cerebral changes in PTSD pathophysiology [3]. The SPS model induces an overexpression of glucocorticoid receptors, low levels of plasma glucocorticoid (which is related to the HPA system), and other cerebral changes, such as hippocampal atrophy, that are similar to changes seen in PTSD patients [7]. The over-suppression of the HPA system has been observed in PTSD patients as a typical phenomenon. The most important advantage of the SPS model in rats is that a single exposure to a stressor can induce long-lasting effects of behavioral and neurological changes as typical symptoms of PTSD [3]. As a result, the rat SPS model is widely used to explore changes in the above symptoms in a PTSD-like pathophysiology [8]. However, the SPS model was established mainly in rats. We hypothesized that there were likely to be no significant differences between rats and mice when PTSD-like symptoms are evaluated.

In this study, we attempted to clarify the applicability of the SPS model of PTSD to mice instead of rats, not only to save space and experimental equipment, but also to enable the use in PTSD research of genetic engineering techniques such as genetic recombination and gene analyses, for which mice are frequently used.

## Materials and Methods

All reagents and chemicals were of the highest commercial grade available. All of the behavioral studies were performed with male ICR mice (Clea Japan, Tokyo, Japan), aged 8-9 weeks. Mice were provided free access to food and water while being housed in groups at constant room temperature ( $24 \pm 1^\circ\text{C}$ ) and humidity (55%) with a 12 h light/12 h dark cycle (lights on at 07:00 h) for 1 week before the experiments. The principles of laboratory animal care and all experimental procedures were in strict accordance with the Guidelines for Animal Experiments of the Shujitsu University School of Pharmacy.

The experimental procedure was modified from a previous method [3]. Briefly, the SPS protocol was con-

ducted in three stages: the mouse are restrained for 2 h and then immediately underwent a 20-min forced swim in 3 L of  $25^\circ\text{C}$  water in a 5-L glass beaker. Following a 15-min recuperation period, the mouse was exposed to ether until it lost consciousness. The mouse was then left in its home cage until the behavioral analysis.

To confirm the pathophysiology of PTSD, we subjected the mice to HPA negative feedback testing at 1, 4, 8, and 12 weeks after the application of the SPS protocol. First, the mice were tested for the SPS-applied effect on a dexamethasone (DEX) suppression of plasma corticosterone to test the effects of the SPS protocol. We divided the mice into four groups: control + saline,  $n=6$ ; control + DEX,  $n=6$ ; SPS + saline,  $n=7$ ; and SPS + DEX,  $n=7$ . The control and SPS-applied mice received a subcutaneous injection of either DEX (0.05 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) or normal sterile saline. Blood was collected via the caudal vein for the measurement of corticosterone. The collected blood samples were centrifuged at 6,000g for 5 min to collect plasma. Plasma corticosterone levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (AssayPro, St. Charles, MO, USA) according to the manufacturer's instructions. Corticosterone data are shown as ng/ml, and were determined in both groups in the presence and absence of DEX.

To investigate PTSD-like characteristics such as those affecting cognition, emotion, and motor activity, we subjected other mice to a light/dark box test (to assess anxiety), a Y-maze test (working memory), a cliff avoidance test (visual cognition), and an open field test (locomotor activity) at 1, 4, 8 and 12 weeks after the application of the SPS protocol. The behavioral analysis was conducted using 16 mice (control:  $n=8$ , SPS:  $n=8$ ) according to a modified form of previous methods [9-11]. Briefly, the light/dark testing was conducted in a box apparatus consisting of a lighted compartment and a dark component, each 40 cm long, 20 cm high, and 40 cm wide: each compartment had an inner runway ( $8 \text{ cm}^2$ ). The mouse was placed in the dark compartment at the start time, and the amount of time that the mouse spent in each compartment during a 5-min observation period was determined (Fig. 1A) [9].

The Y-maze apparatus (Muromachi Kikai, Tokyo, Japan) was based on a black-painted plastic board. Each arm was 40 cm long, 10 cm high, 4 cm wide at the bottom, and 10 cm wide at the top. The arms converged to

an equilateral triangular central area that was 4 cm at its longest axis. The mouse was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The mouse was considered to have entered an arm when it had placed its hind paws completely inside it. Alternation was defined as successive entries into the three arms, on overlapping triplet sets of test. The alternation ratio was calculated as the ratio of actual to possible alternation (defined as the total number of arm entries minus 2) multiplied by 100 (Fig. 1B) [10].

To examine visual cognition, we devised a new evaluation method, “the cliff avoidance test”. The apparatus of the cliff avoidance test consisted of 3 areas: an open area (a transparent floor; 30 cm×30 cm×15 cm), a bench area (a black floor surrounded with a transparent

wall 10 cm in height; 30 cm×30 cm×15 cm), and a start area (10 cm×4 cm×7 cm) (Fig. 1C). The mouse was placed in the start area at the start time, and then the amount of time spent in each of the 3 areas was recorded using video. We used the time spent in the open area as an index of anxiety-related behavior during each 5-min observation. (Unpublished data).

In the open field test, the mouse was placed on the center of a Hall’s-type open field apparatus (60 cm dia., 50 cm high), and the number of quadrants it entered within a 5-min period was counted; the result was then regarded as a measure of locomotor activity (Fig. 1D) [11].

The results are presented as means±standard error of the mean (SEM). The significance of differences between groups was tested using a two-way analysis of variance (ANOVA) (two experimental groups and four time points) with repeated measures. A *p*-value<0.05 was accepted as denoting a statistically significant difference. All analyses were performed with statistical analysis software (SPSS ver. 21, SPSS, Chicago, IL, USA).

### Results

Control and SPS-applied mice were tested for a DEX suppression of plasma corticosterone at 1, 4, 8 and 12 weeks after the application of the SPS protocol. The plasma corticosterone levels of the DEX-treated control and DEX/SPS-applied mice were significantly lower than those of the 2 vehicle-treated groups at all four-time points after the SPS protocol (Table 1). A two-way ANOVA (the 4 groups×the 4 time points) with repeated measures showed significance in all three main effects: group ( $F_{1, 20}=67.383, p<0.01$ ), time ( $F_{3, 20}=60.431, p<0.01$ ), and group × time ( $F_{3, 20}=8.707, p<0.01$ ).

Moreover, the application of the SPS protocol significantly reduced ratio of DEX- to saline-treated corticosterone levels at both 8 and 12 weeks compared to the ratio of DEX- to saline-treated corticosterone levels

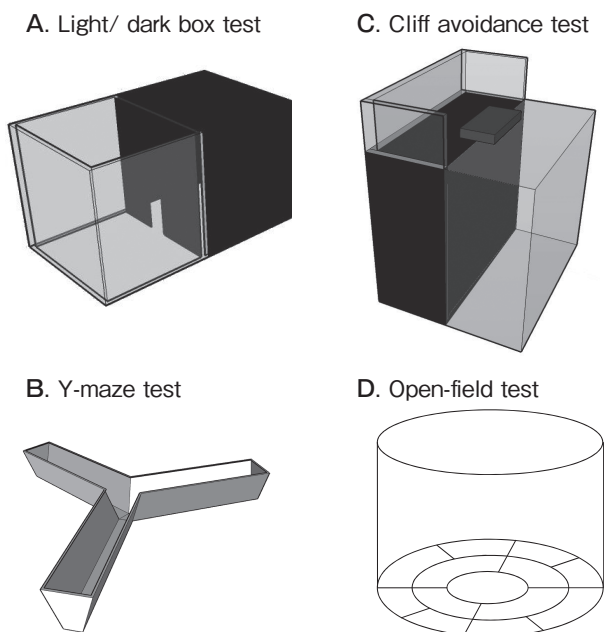


Fig. 1 The apparatuses used in the (A) light/dark box test, (B) Y-maze test, (C) cliff avoidance test, and (D) open-field test.

Table 1 Time course of changes in serum corticosterone levels

	1 week	4 weeks	8 weeks	12 weeks
Vehicle-treated control mice	190.5 ± 15.6	164.3 ± 18.8	153.0 ± 16.6	153.1 ± 14.9
DEX-treated control mice	29.2 ± 9.0*#	28.2 ± 7.6*#	25.3 ± 6.2*#	26.0 ± 8.4*#
Vehicle-treated SPS-applied mice	169.3 ± 3.8	164.3 ± 16.8	164.4 ± 16.6	140.8 ± 10.3
DEX-treated SPS-applied mice	21.7 ± 3.5*#	15.4 ± 2.7*#	11.3 ± 2.1*#	7.7 ± 1.2*#

Each value is the mean ± SEM of 6 mice.

\**p*<0.05 vs. the relative Vehicle-treated control group.

#*p*<0.05 vs. the relative Vehicle-treated SPS-applied group.

between the control and SPS-applied mice (Table 2). Thus, the application of the SPS protocol induced an over-suppression of the HPA system by DEX treatment in mice.

In the light/dark box test, the amount of time that the SPS-applied mice spent in the lighted compartment of the light/dark box was significantly lower at 8 and 12 weeks after the application of SPS (Fig. 2A). A two-way ANOVA (2 groups  $\times$  4 time points) with repeated measures showed significance in all 3 main effects: group ( $F_{1, 56}=21.933$ ,  $p<0.01$ ), time ( $F_{3, 56}=4.903$ ,  $p<0.01$ ), and group $\times$ time ( $F_{3, 56}=4.872$ ,  $p<0.01$ ).

In the Y-maze test, the SPS-applied mice tended to have a lower alternation ratio at 12 weeks after the application of the SPS protocol compared with the control mice, but the difference was not significant

**Table 2** Time course of changes in the ratio of DEX to vehicle in serum corticosterone levels

	1 week	4 weeks	8 weeks	12 weeks
Control mice	16.8 $\pm$ 5.2	18.0 $\pm$ 4.9	17.5 $\pm$ 4.3	17.8 $\pm$ 5.8
SPS-applied mice	11.8 $\pm$ 2.2	9.6 $\pm$ 1.6	7.0 $\pm$ 1.3*	5.9 $\pm$ 0.8*

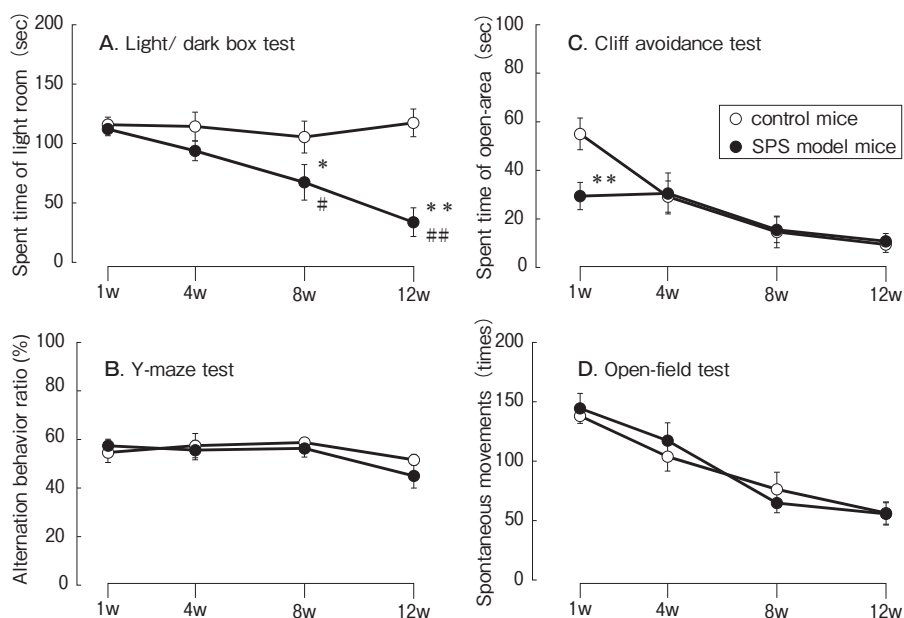
Each value is the mean  $\pm$  SEM of 6 mice.

\* $p<0.05$  vs. the relative control group.

(Fig. 2B). A two-way ANOVA (2 groups  $\times$  4 time points) with repeated measures did not reveal a significant difference in any of the 3 main effects: group ( $F_{1, 56}=0.562$ ,  $p>0.05$ ), time ( $F_{3, 56}=2.571$ ,  $p>0.05$ ), and group $\times$ time ( $F_{3, 56}=0.530$ ,  $p>0.05$ ).

In the cliff avoidance test, the SPS-applied mice spent significantly less time in the open area only at 1 week after the SPS protocol (Fig. 2C), although at 4 weeks, both the control and SPS-applied groups had gradually and time-dependently spent less time in the open area. A two-way ANOVA (2 groups  $\times$  4 time points) with repeated measures showed a significant main effect of time ( $F_{3, 56}=12.267$ ,  $p<0.01$ ), but not of group ( $F_{1, 56}=1.765$ ,  $p>0.05$ ), or of group  $\times$  time ( $F_{3, 56}=2.582$ ,  $p>0.05$ ).

In the open field test, the SPS-applied mice tended to show reduced locomotor activity in a time-dependent manner until 12 weeks after the application of the SPS protocol (Fig. 2D). A two-way ANOVA (2 groups  $\times$  4 time points) with repeated measures showed a significant main effect of time ( $F_{3, 56}=23.511$ ,  $p<0.01$ ), but not of group ( $F_{1, 56}=0.057$ ,  $p>0.05$ ), or of group  $\times$  time ( $F_{3, 56}=0.444$ ,  $p>0.05$ ).



**Fig. 2** Time course of the changes in the (A) light/dark box test, (B) Y-maze test, (C) cliff avoidance test, and (D) open-field test after the application of the SPS protocol. Each value is the mean  $\pm$  SEM of 8 mice.

\* $p<0.05$ , \*\* $p<0.01$  vs. the relative control group.

## Discussion

The SPS protocol consists of three stresses: restraint, forced swimming, and ether anesthesia, which correspond to psychological, physiological, and endocrinological stress, respectively. The sequence of stresses in the SPS model is somewhat arbitrary and does not simulate the common set of traumas experienced by patients with PTSD (*i.e.*, the protocol lacks ecological validity). However, each of the three stresses markedly increased the serum corticosterone levels of the mice, and by combining the stresses, it seemed that the SPS model could achieve a severity of symptoms similar to that experienced by patients with PTSD such as various HPA-axis abnormalities. The present SPS protocol allows us to avoid the risk that habituation processes will diminish the effect of a stressor on a mouse's behavior. We also observed that the application of this protocol could lead to enhanced negative feedback of the HPA-axis 7 days after its application, through time-dependent sensitization (TDS). This endocrinological characteristic, which has been consistently replicated in the rat SPS model, is one of the advantages of the SPS protocol over other animal models of PTSD [2, 3].

In the present study, the application of the SPS protocol significantly reduced the ratio of DEX- to saline-treated corticosterone levels at 8 and 12 weeks, but not at 1 week (7 days). However, several previous reports suggested that an over-suppression of the HPA system by DEX was correlated with the onset of behavioral abnormalities including those observed in SPS-applied rats tested in a light/dark box [2, 12]. Our present findings demonstrated that the application of the SPS protocol in mice also induced an over-suppression of the HPA system by DEX treatment similar to that in rats, although there are several differences between the mouse SPS model and the rat SPS model. In a rat study, it was proposed that the use of an undisturbed period is a necessary condition to produce PTSD-like manifestations [2].

Our present results indicated that marked SPS-induced behavioral alterations might be recognized at 8-12 weeks after the application of the SPS protocol, at least in the light/dark box test, which is commonly used to evaluate anxiety-related behavior in rodents. This test is based on rodent's innate aversion to brightly illuminated areas and on the spontaneous exploratory behav-

ior of rodents in response to mild stressors such as a novel environment and light [4]. Thus, our present light/dark box test results suggest that the application of the SPS protocol in mice mainly induced anxiety-related behavior at >8 weeks after the protocol's application, similar to the findings in rats, although there are several differences between the mouse and rat SPS models [2]. Our application of the SPS protocol reduced the time that the SPS-applied mice spent in the light compartment but not in the dark compartment, in a time-dependent manner until 12 weeks after the SPS protocol's application. We therefore speculate that the application of the SPS protocol may emphasize PTSD-like anxiety. We also suspect that mice become habituated to this light/dark box test because the control mice did not display a reduced latency to move from the light to the dark compartment of the test box.

We also observed that SPS-induced behavioral alterations in the mice in the cliff avoidance test only at 1 week after the SPS application, not at 8-12 weeks. In the previous SPS-applied rat study, we did not confirm to evaluate the effect of the SPS protocol on animal's behavior in the cliff avoidance test. This test, which is based on the cliff-avoidance reaction, is an index of behavioral teratology in rodents which can be impaired by motor, arousal, or cognitive dysfunction. As the cliff-avoidance reaction is thought to represent a natural tendency of animals to avoid a potential fall from a height, the impairment of the cliff-avoidance reaction means that an aspect of maladaptive impulsive behaviors in mature rodents may result from deficient behavioral inhibition [13]. The cliff avoidance test is also based on the visual cliff avoidance task, which assesses complex visual discrimination learning [14].

Thus, our results showing that the SPS-applied mice spent less and less time in an open area 1 week after the SPS may indicate that anxiety-related behavior induced by the SPS protocol such as cognitive or impulsive behaviors could be recognized prior to true PTSD-like symptoms. In addition, the results of the SPS application on the open field test results in mice may indicate no effect of the SPS protocol or habituation to this test, because both the control and SPS-applied mice tended to show reduced locomotor activity in a time-dependent manner until 12 weeks, although there was no effect on the locomotor activity of the SPS-applied rats at least until 2 weeks [15].

Animal models that have 3 types of validity are



desired: phenomenological similarity (face validity), corresponding theoretical explanatory frameworks (construct validity), and the ability to predict that a pharmacological agent with efficacy demonstrated in animal studies will have a therapeutic effect in humans (predictive validity) [16]. According to previous reports, the rat SPS model had at least the face and construct validities as an animal model of PTSD [2]. However, the limitations of the rat SPS model are as follows: (1) the stressor does not produce PTSD-like symptoms in a dose-dependent manner, (2) reduced responsiveness to the stressor has not been well studied, and (3) the degree of inter-individual variability in response to stressors is still unknown [2]. In addition, when attempting to establish a rodent PTSD model, one must consider some differences in behavioral characteristics between rats and mice, such as their anxiety-related behavior, and it should be noted that different experimental methods have often been used in rats and mice.

Our present study provides data that support currently known mechanisms relevant to PTSD because the development of a similarly validated mouse model emphasizes the benefits and cross-species utility of rodent PTSD models; for example, previous studies suggested that characteristics of a mouse SPS model are relevant to PTSD and that severe, multimodal stress modifies fear learning in mice, although strains other than ICR (as in the present study) such as Swiss or C57BL/6 mice [8, 17]. However, our present experiments revealed that the application of the SPS protocol did not significantly change the alternation behavior ratio compared with the control mice in the Y-maze test, since the Y-maze test examined mainly the entire working memory rather than only fear memory. In contrast, the disruption of spatial memory in addition to fear memory in SPS-applied was reported; nevertheless, it should be kept in mind that the details of the differences between the characteristics of mice and rats as animal models of PTSD are not completely known [18].

Our present findings suggest that the investigation of behavioral abnormalities, mainly regarding cognitive or impulsive behaviors revealed in the cliff avoidance test, may be useful for finding indications of a PTSD-like phenomenon. However, further studies are needed to evaluate the validity of the SPS mouse model, for both analyses of the pathophysiology of PTSD and investigations of therapeutic drugs to treat PTSD-like symptoms.

In particular, we expect that the time point of 12 weeks after the application of the present SPS protocol could be useful for animal models of PTSD.

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