Effects of single prolonged stress and D-cycloserine on contextual fear extinction and hippocampal NMDA receptor expression in a rat model of PTSD

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Abstract Although the impaired extinction of traumatic memory is one of the hallmark symptoms of PTSD, the underlying mechanisms of impaired extinction are unclear and effective pharmacological interventions have not yet been developed. Single prolonged stress (SPS) has been proposed as an animal model of PTSD, since rats subjected to SPS (SPS rats) show enhanced negative feedback of the HPA axis and increased contextual fear, which are characteristics similar to those observed in patients with PTSD. In this study, using SPS rats, we examined (a) the ability of SPS to impair fear extinction, (b) whether D-cycloserine (DCS) can alleviate impaired fear extinction in SPS rats, and (c) the effect of SPS and/or DCS on the levels of N-methyl-D-aspartate (NMDA) receptor subunit mRNAs in the rat hippocampus during extinction training. SPS rats exhibited impaired fear extinction in the contextual fear test, which was alleviated by the repeated administration of DCS. The effect of enhanced extinction, induced by the administration of DCS to SPS rats, was maintained for one week following extinction training. SPS induced significant upregulation of the levels of NMDA receptor subunit mRNAs before and during the period of extinction training, while repeated administration of DCS eliminated the enhanced mRNA levels of NMDARs. Behavioral analyses indicated that SPS is an appropriate animal model of PTSD and that DCS may be effective in the treatment of PTSD. These findings suggest that DCS, irrespective of its mechanistic involvement in the enhancement of fear extinction, may help to reverse

hippocampal plasticity, and thus reverse the NMDA compensatory alterations.

Keywords: posttraumatic stress disorder (PTSD); single prolonged stress (SPS); extinction; contextual fear conditioning; NMDA receptor subunits (NMDARs); D-cycloserine (DCS)

INTRODUCTION

Posttraumatic stress disorder (PTSD), an anxiety disorder is induced by exposure to life-threatening trauma. According to the DSM-IV diagnostic criteria, the characteristic features of PTSD include persistent reexperiencing trauma, avoidance and numbing, and hyperarousal (American Psychiatric Association 1994). The majority of patients with PTSD exhibit long-lasting reexperience of traumatic events and subsequently avoid the stimuli that link traumatic events, even though they recognize that the traumatic event is no longer occurring. Recent advances in our understanding of the mechanisms underlying fear extinction has led to the hypothesis that dysfunctional fear extinction plays an important role in the development of clinical symptoms such as reexperiencing of trauma in PTSD, since extinction is defined as a reduction in conditioned fear response when the conditioned stimulus is repeatedly presented in the absence of an unconditioned stimulus (Milad *et al*, 2006; Quirk *et al*, 2006; Rauch *et al*, 2006; Rothbaum and Davis, 2003).

Although the neurocircuitry mechanism of contextual fear extinction is not fully understood, brain regions such as the hippocampus, amygdala, and prefrontal cortex are involved in the memory extinction (Sotres-Bayon *et al*, 2004; Sotres-Bayon *et al*, 2006). Likewise, though the molecular mechanism of contextual fear extinction remains to be precisely determined, the N-methyl-D-aspartate (NMDA) receptor appears to play a critical

role in fear extinction (Falls et al, 1992; Ledgerwood et al, 2003; Ledgerwood et al, 2004; Ledgerwood et al, 2005; Rodrigues et al, 2001; Santini et al, 2001; Walker et al, 2002). In addition to amygdaloid NMDA receptors, emerging evidence has recently indicated that hippocampal NMDA receptors (NMDARs) and subsequent signaling pathways are involved in the mechanism for extinction of contextual fear (Bevilaqua *et al*, 2005; Szapiro *et al*, 2003). For example, Szapiro and colleagues, using a step-down avoidance paradigm, found that intra-hippocampal infusions (CA1 region) of the NMDA receptor antagonist, AP5, immediately after the first of four daily extinction exposures, produced a lasting impairment that persisted even with additional extinction training without infusions (Szapiro *et al*, 2003).

With regard to facilitatory effects of pharmacological agents on fear extinction, recent studies with rats have indicated that the extinction of particular fear responses, such as freezing (Ledgerwood *et al*, 2003; Ledgerwood *et al*, 2004; Ledgerwood *et al*, 2005) and fear potentiated startle (Walker *et al*, 2002), can be enhanced with the glycine partial agonist D-cycloserine (DCS). DCS enhances excitatory neurotransmission mediated by NMDA receptors, by binding to the strychnine-insensitive glycine recognition site of the NMDA receptor complex, without inducing neurotoxicity. Indeed, in clinical studies, Ressler et al. (2004) and Hofmann et al. (2006) have recently reported that DCS facilitates fear extinction in patients with anxiety disorders.

On the other hand, development of not only an effective treatment but also appropriate animal model will promote our understanding of mechanisms of PTSD. For instance, Liberzon et al. proposed a rat model of PTSD involving single prolonged stress (SPS) (Liberzon *et al*, 1997; Liberzon *et al*, 1999). Rats subjected to SPS (SPS rats) show enhanced negative feedback of the hypothalamic-pituitary-adrenal (HPA) axis function in response to glucocorticoid administration (Liberzon *et al*, 1997; Liberzon *et al*, 1999). In addition, SPS rats also exhibit a sustained exaggeration of the acoustic startle response (Khan and Liberzon, 2004). Furthermore, we recently demonstrated that SPS rats exhibit enhanced contextual freezing, increased anxiety-like behavior in the elevated plus maze, and stress-induced analgesia compared to rats subjected to sham treatment (Imanaka *et al*, 2006; Takahashi *et al*, 2006). Since these behavioral responses seen in rats subjected to SPS resemble the clinical symptoms seen in patients with PTSD, it is postulated that SPS is an appropriate animal model of PTSD.

In this context, we first examined whether dysfunctional extinction of contextual fear was found in SPS rats. This would allow us to elucidate the involvement of dysfunctional extinction in the pathophysiology of PTSD. Secondly, we examined the effect of DCS on the dysfunctional extinction of contextual fear. We then examined alterations in the levels of mRNA coding for NMDA receptor subunits, including NR1, NR2A, NR2B, NR2C, in the

hippocampus during extinction training, and the effects of DCS on NMDAR mRNA levels in the hippocampus during extinction training.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing between 300 g and 350 g (Japan Charles River, Yokohama, Japan) were used in our studies. 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. A total of 176 rats were used in this study (Experiment 1: N = 20, Experiment 2: N = 76, Experiment 3: N = 44, Experiment 4: N = 36). All animal procedures were conducted in strict accordance with the Hiroshima University School of Medicine Animal Care Committee Guiding Principles on Animal Experimentations in Research Facilities for Laboratory Animal Science, School of Medicine, Hiroshima University.

Single Prolonged Stress (SPS)

According to the method of Liberzon et al. (Liberzon *et al*, 1997; Liberzon *et al*, 1999), SPS was conducted in three stages: restraint for 2 hr, forced swim for 20 min, and ether anesthesia. Each rat was restrained for 2 hr by placing it inside a disposable clear polyethylene cone bag (Asahikasei, Tokyo, Japan) with only the tail protruding (Suenaga *et al*, 2004). The large end of the cone was closed with tape at the base of the tail. The bag size was

adjusted according to the size of the rat in order to achieve complete immobilization. A hole in the small end of the cone allowed the rats to breathe freely. 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, and then left undisturbed in their home cage for 7 days.

Contextual fear conditioning and extinction training

In the first experiment, we investigated influence of SPS on fear extinction (Experiment 1). Animals were randomly assigned to two groups (SPS, Sham). Sham rats were left alone without handling in their cages. During SPS treatment, Sham rats were moved and placed in an identical cage where SPS was conducted. Rats were placed in a conditioning chamber (325 width × 280 height × 500 depth mm), and then were exposed to a 180-sec conditioning context without any stimulation (i.e., a tone). Immediately after that, they received a 4-sec, 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 intertrial interval of 30 sec. Following the footshock, 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 apparatus) in the absence of footshock. Twenty-four hours after fear conditioning, rats were placed for 10 min without footshock in the same chamber where the footshock was delivered. In a similar manner, extinction training was performed on each of 5 consecutive days following fear conditioning (Fig. 1). Freezing was monitored using a time sampling method in which each rat was observed once every 5 sec and a percentage score was calculated for the proportion of the total observation period spent freezing. Freezing was defined as the total absence of body or head movement except for that associated with breathing. Freezing behavior of the rat was recorded on videotape, and later scored blindly by well-trained experimenters. Pearson's correlation coefficient was calculated to examine the inter-rater reliability. The inter-rater reliability between the two scorers was high ($r = 0.96$).

Drug treatment

In this experiment, we investigated the effects of DCS on fear extinction. DCS was purchased from Sigma-Aldrich (St. Louis, MO, USA). To avoid stress from the injection per se and to mimic clinical use, we administered DCS orally. For DCS administration, DCS was administered daily for 6 days (from the end of fear conditioning to the beginning of the fifth extinction training) (Experiment 2A).

Animals were randomly assigned to 4 groups (SPS, SPS + DCS, Sham, Sham + DCS). In addition, to examine the sustained effect of DCS upon fear extinction, we evaluated the freezing responses of animals from each of the 4 groups one week after the fifth extinction training period, in the absence of DCS. The two groups treated with DCS (SPS + DCS, Sham + DCS) were not treated with DCS between the fifth extinction training period and day 12 (Experiment 2B). Milli-Q water was available freely for the SPS and Sham groups, and DCS dissolved in Milli-Q water was available freely for the SPS + DCS and Sham + DCS groups. The DCS dose (15 mg/kg per rat) was chosen on the basis of the results of other behavioral studies (Ledgerwood *et al*, 2003; Ledgerwood *et al*, 2005; Walker *et al*, 2002). Prior to the present study, we measured the consumption of drinking water in our animal facility. Specifically, we measured the reduction of water volume in the animal's water-bottles each day for 6 consecutive days in order to verify the intake of DCS. Based upon this measurement, we found that three rats (B.W. of each rat was approximately 330 g at the beginning of measurement) in one cage consumed approximately 120 ml of Milli-Q water per day (N = 54). Thus, the average weight of a rat (330 g), was used to calculate the appropriate dose of DCS (15 mg/kg × average weight of 3 rats × 6). The dose was dissolved in 240 ml of Milli-Q water. DCS solution (240ml) was added to the water-bottle in each cage at the beginning of DCS administration (supplementary Fig. 1). During the present study, we also measured the weight

of each rat daily and adjusted the concentration of DCS to 15 mg/kg per rat each day, based upon the average weight of three rats in one cage. We also verified that the three rats in any one cage consumed approximately 120 ml of Milli-Q water, with or without DCS. Fear conditioning and extinction training were performed as described above.

Measurement of the levels of NMDARs in the hippocampus by real-time quantitative polymerase chain reaction (RT-PCR)

To elucidate whether the hippocampal glutamatergic systems are involved in fear extinction, we used RT-PCR to measure alterations in NMDAR mRNA levels in the hippocampus during extinction training (Experiment 3). Animals were randomly assigned to two groups (SPS, Sham), and were sacrificed by decapitation at the indicated time on day 0 (before fear conditioning), on day 1 (after the first context exposure), or on day 4 (after the fourth context exposure). NMDAR mRNA levels were analyzed between the two groups and then compared across experimental time-points (days). Further, we investigated the effects of repeated DCS administration on the NMDAR mRNA levels in the hippocampus during extinction training (Experiment 4). DCS was repeatedly administered for 5 days (from the end of fear conditioning to the beginning of the fourth extinction training). Animals were randomly assigned to 4 groups (SPS, Sham, SPS + DCS, and Sham + DCS), and were

sacrificed by decapitation after the fourth context exposure. Hippocampal tissue was removed from the brain and quickly frozen using powdered dry ice and stored at -80°C. Total RNA was extracted using RNAqueous™ Total RNA Isolation kits (Ambion, Austin, TX, USA) according to the manufacturer's instructions, and then a single-stranded cDNA was synthesized using the QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany), which provided a procedure for genomic DNA elimination and reverse transcription. RT-PCR was performed with an ABI7700 sequence detection system (PE Applied Biosystems, Foster City, CA, USA) to quantify relative mRNA levels in samples. RT-PCR was performed to amplify the mRNA of NMDARs. The primers and TaqMan hybridization probes were designed using Primer Express software (PE Applied Biosystems). Table 1 shows the sequences and fluorescent dyes of the PCR primers and TaqMan probes for each molecule. The TaqMan probe, which was designed to hybridize to the PCR products, was labeled with a fluorescent reporter dye at the 5'end and a quenching dye at the 3' end. PCR was carried out with TaqMan Universal PCR Master Mix (PE Applied Biosystems). All standards and samples were assayed in triplicate. Thermal cycling was initiated with an initial denaturation at 50°C for 2 min and 95°C for 10 min. After this initial step, 40 cycles of PCR were performed. Each PCR cycle consisted of heating at 95°C for 15 s for melting and 60°C for 1 min for annealing and extension. The PCR assay for glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) was performed using the TaqMan Rodent GAPDH Control Reagents kit (PE Applied Biosystems). The mRNA levels of NMDARs were detected by RT-PCR (ABI PRISM 7700 sequence detection system) and the ratio of the concentration of the target molecule to that of GAPDH (target molecule/GAPDH) in unknown samples was calculated. For reference, the RT-PCR standard curve plot and the amplification plot of NR1 before fear conditioning are shown (supplementary Fig. 2 and 3).

Statistical Analysis

All values shown represent the mean ± SEM. In Experiment 1, freezing responses of the Sham and SPS groups were compared by two-way ANOVA (stress, day) for repeated measures (day). In order to further evaluate effects of SPS on each experimental day, we used the unpaired Student's t test. In Experiment 2, freezing responses of the 4 groups were compared by three-way ANOVA (i.e., stress, drug, day) for repeated measures (day). In order to evaluate the effect of DCS upon the consolidation of original fear in Experiment 2A, we used two-way ANOVA (stress, drug) to analyze the freezing responses of animals from the 4 groups on day 1. In Experiment 3, mRNA levels were analyzed using the unpaired Student's t test or by two-way ANOVA (stress, day) followed by appropriate post-hoc comparisons. Post-hoc comparisons were performed using Tukey's test. In Experiment 4, data for mRNA levels were

analyzed by two-way ANOVA (stress, drug). Results were considered statistically significant at $P < 0.05$.

RESULTS

Experiment 1: Influence of SPS on fear extinction

In Experiment 1, two-way repeated measures ANOVA showed a significant main effect of day [$F(4, 72) = 34.01$, $p < 0.01$], stress [$F(1, 18) = 20.14$, $p < 0.01$], and an interaction between stress and day [$F(4, 72) = 2.77$, $p < 0.05$]. In order to further evaluate the effects of SPS on each experimental day, we used the unpaired Student's t test. The freezing responses on day 1 did not differ between the SPS and Sham groups [$t(18) = 1.54$, $p = 0.13$], suggesting that contextual fear conditioning was successful in both groups of rats. During repeated context exposure, however, a significant difference in the freezing responses between the two groups appeared on day 2, and significant differences persisted until day 5 [day 2: $t(18) = 2.76$; $p < 0.05$, day 3: $t(18) = 3.67$; $p < 0.01$, day 4: $t(18) = 3.63$; $p < 0.01$, day 5: $t(18) = 4.11$; $p < 0.01$] (Fig. 2).

Experiment 2: Effects of DCS administration on fear extinction

In Experiment 2A, three-way repeated measures ANOVA demonstrated significant main effects of day [$F(4, 72) = 105.39$, $p < 0.01$], stress [$F(1, 18) = 16.41$, $p < 0.01$], drug [$F(1, 18) = 9.81$, $p < 0.01$], and significant interactions between stress and drug [$F(1, 18) = 5.71$, $p < 0.05$], and between stress and day [$F(4, 72) = 2.71$, $p < 0.05$]. There were no significant

interactions between stress and drug and day [F (4, 72) = 1.26, p = 0.29], or between drug and day [F (4, 72) = 1.02, p = 0.39] (Fig. 3). In order to evaluate the effect of DCS upon the consolidation of original fear, we used two-way ANOVA to analyze the freezing responses of animals from each of the 4 groups on day 1. Two-way ANOVA revealed no significant effects associated with stress [F (1, 36) = 1.91.1, p = 0.17] or drug [F (1, 36) = 1.93.1, p = 0.17], and revealed no significant interaction between stress and drug [F (1, 36) = 0.29.1, p = 0.59]. Collectively, these data suggest that DCS did not affect the consolidation of original fear.

In Experiment 2B, we examined the sustained effect of DCS upon enhanced extinction. In this experiment, rats were not treated with DCS between the fifth period of extinction training and day 12. Both of the groups administered with DCS exhibited less freezing on day 12 as compared to day 5. Three-way repeated measures ANOVA revealed significant main effects associated with day [F (1, 16) = 20.71, p < 0.01], stress [F (1, 16) = 21.39, p < 0.01] and drug [F (1, 16) = 15.6, p < 0.01]. Further analysis revealed significant interactions between stress and drug [F (1, 16) = 7.43, p < 0.05], between stress and day [F (1, 16) = 9.42, p < 0.01], and between drug and day [F (1, 16) = 11.92, p < 0.01]. There was no significant interaction between stress and drug and day [F (1, 16) = 2.66, p = 0.12] (Fig. 4).

Experiment 3: Measurement of the mRNA levels of NMDARs in the hippocampus during

extinction training

In Experiment 3, a total of 44 rats were used (day 0: Sham; N = 6, SPS; N = 6, day 1: Sham; N = 7, SPS; N = 7, day 4: Sham; N = 9, SPS; N = 9). Rats showed freezing responses similar to comparable rats in Experiment 1. This was despite the fact that different sets of rats were used for each experiment.

For comparison, when we compared the SPS and Sham groups, before fear conditioning, we observed that the levels of all NMDAR subunit mRNAs in the SPS group were higher than those in the Sham group [NR 1: $t(10) = 5.04$; $p < 0.01$, NR2A: $t(10) = 2.93$; $p < 0.05$, NR2B: $t(10) = 3.68$; $p < 0.01$, NR2C: $t(10) = 5.11$; $p < 0.01$].

The results after the first context exposure were similar to the results observed before fear conditioning; i.e., the levels of all NMDAR subunit mRNAs in the SPS group were also higher than those in the Sham group [NR 1: $t(12) = 3.27$; $p < 0.01$, NR2A: $t(12) = 3.92$; $p < 0.01$, NR2B: $t(12) = 3.95$; $p < 0.01$, NR2C: $t(12) = 4.20$; $p < 0.01$].

After the fourth context exposure, the levels of all NMDAR subunit mRNAs in the SPS group were still higher than those in the Sham group [NR 1: $t(16) = 4.81$; $p < 0.01$, NR2A: $t(16) = 2.97$; $p < 0.01$, NR2B: $t(16) = 3.53$; $p < 0.01$, NR2C: $t(16) = 5.63$; $p < 0.01$].

When using two-way ANOVA to compare the levels of NMDAR mRNAs across the experimental timepoints, we revealed significant effects of stress [$F(1, 38) = 56.73$, $p < 0.01$]

and day [F (2, 38) = 41.56, p < 0.01] upon NR1 mRNA levels. We found no significant interaction between stress and day [F (2, 38) = 1.23, p = 0.3]. Post-hoc analysis across experimental timepoints, revealed that there were significant differences between day 0 and day 1, and between day 0 and day 4 (p < 0.01, respectively) (Fig. 5A). For NR2A mRNA levels, two-way ANOVA detected significant effects of stress [F (1, 38) = 22.18, p < 0.01] and day [F (2, 38) = 7.78, p < 0.01]. No significant interaction between stress and day was found [F (2, 38) = 1.2, p = 0.31]. Post-hoc analysis revealed that there was a significant difference between day 0 and day 1 (p < 0.01) when comparing experimental timepoints (Fig. 5B). For NR2B mRNA levels, two-way ANOVA detected significant effects of stress [F (1, 38) = 35.39, p < 0.01] and day [F (2, 38) = 6.45, p < 0.01]. No significant interaction was found between stress and day [F (2, 38) = 0.39, p = 0.67]. Post-hoc analysis revealed that there was a significant difference between day 0 and day 1 (p < 0.01) across experimental timepoints (Fig. 5C). For NR2C mRNA levels, two-way ANOVA detected significant effects of stress [F (1, 38) = 74.38, p < 0.01] and day [F (2, 38) = 22.07, p < 0.01]. No significant interaction was detected between stress and day [F (2, 38) = 2.12, p = 0.13]. Post-hoc analysis across experimental timepoints revealed that there were significant differences between day 0 and day 1, and between day 0 and day 4 (p < 0.01, respectively) (Fig. 5D).

Collectively, our comparison of NMDAR expression in the hippocampus between the

SPS and Sham groups demonstrated that the levels of all 4 NMDAR subunits in the SPS group were significantly higher than those in the Sham group, both before and during the entire period of extinction training. In addition, in comparing data across the experimental timepoints, we have demonstrated that extinction training affected the levels of all NMDAR subunit mRNAs in the hippocampus.

Experiment 4: Effects of repeated DCS administration on NMDAR mRNA levels in the hippocampus during extinction training

In Experiment 4, a total of 36 rats were used (N = 9 per group). Rats showed freezing responses similarly to comparable rats in Experiment 2A. This was despite independent sets of rats being used for each experiment.

In the analysis of NR1 mRNA levels, two-way ANOVA showed a significant effect of stress [$F(1, 32) = 9.33$, $p < 0.01$], and a significant interaction between stress and drug [$F(1, 32) = 8.47$, $p < 0.01$]. No significant effect of drug was found [$F(1, 32) = 1.22$, $p = 0.27$] (Fig. 6A). For NR2A mRNA levels, two-way ANOVA showed a significant effect of drug [$F(1, 32) = 7.34$, $p < 0.05$] and a significant interaction between stress and drug [$F(1, 32) = 10.4$, $p < 0.05$]. No significant effect of stress was found [$F(1, 32) = 2.33$, $p = 0.13$] (Fig. 6B). For NR2B mRNA levels, two-way ANOVA showed a significant effect of drug [$F(1, 32) = 31.91$,

$p < 0.01$] and a significant interaction between stress and drug [$F(1, 32) = 6.11$, $p < 0.05$]. No significant effect of stress was found [$F(1, 32) = 3.71$, $p = 0.06$] (Fig. 6C). For NR2C mRNA levels, two-way ANOVA showed a significant effect of stress [$F(1, 32) = 5.76$, $p < 0.05$] and a significant interaction between stress and drug [$F(1, 32) = 12.1$, $p < 0.01$]. No significant effect of drug was found [$F(1, 32) = 2.36$, $p = 0.13$] (Fig. 6D).

Collectively, the statistical analyses revealed that DCS led to a reduction of mRNA levels that was most consistent for the SPS group.

DISCUSSION

Using a contextual fear conditioning paradigm in rats, we examined the influence of SPS on fear extinction, and the effects of DCS on the impaired fear extinction induced by SPS. We then examined alterations in the levels of NMDAR mRNAs in the hippocampus during extinction training and the effects of DCS on NMDAR mRNA levels in the hippocampus during extinction training. The principal findings of this study are that: (1) SPS induced marked impairment of contextual fear extinction in the rats, (2) repeated administration of DCS alleviated impaired fear extinction in SPS rats, and enhanced extinction induced by the administration of DCS was maintained for one week after extinction training, (3) the levels of mRNAs coding for all 4 subunits of the NMDAR in the hippocampus in SPS rats were higher than those in Sham rats during the entire period of extinction training, (4) repeated administration of DCS eliminated the enhanced mRNA levels of NMDARs in the rat hippocampus found 7 days after SPS.

In Experiment 1, SPS rats showed significant impairment of contextual fear extinction as compared with Sham rats. Whereas there was no significant difference in the freezing levels between SPS and Sham rats 24 hours after the fear conditioning, the freezing levels in SPS rats were significantly higher than those in Sham rats after the second extinction training. We recently found no significant difference in locomotor activities between SPS and Sham rats

(Imanaka *et al*, 2006; Takahashi *et al*, 2006). In addition, we reported that the freezing response in SPS rats is not due to abnormalities of sensory-motor function (Takahashi *et al*, 2006).

Since an initial report by Walker et al. (Walker *et al*, 2002), a growing body of evidence has indicated that DCS administration enhances the extinction of conditioned fear (Ledgerwood *et al*, 2003; Ledgerwood *et al*, 2004; Ledgerwood *et al*, 2005; Parnas *et al*, 2005; Woods and Bouton, 2006). In Experiment 2, we observed that repeated DCS administration alleviated the impaired extinction of contextual fear in SPS rats. This effect was also found 7 days after the fifth extinction training. In contrast, the enhanced effect of repeated DCS administration upon extinction was not observed in Sham rats. One possible explanation for this result is that the duration of context exposure (10 min) may be longer for Sham rats. Since the freezing levels in Sham rats decreased relatively rapidly, it is possible that we were not able to ascertain the enhanced effect of DCS (i.e., a floor effect). Another explanation is that, owing to the limitations of our administration procedure, it is possible that each rat failed to consume an adequate amount of Milli-Q water containing DCS.

Our results are generally in agreement with the results of Walker et al. (2002) and Ledgerwood et al. (2003), in which DCS systemic administration enhanced the extinction of conditioned fear. However, since intra-amygdala, as well as systemic administration of DCS

enhanced the extinction of conditioned fear, it is postulated that the amygdala plays an important role in the DCS enhancement, even more so than the hippocampus. In this context, it cannot be ruled out that the oral administration of DCS in the present study affected NMDAR function in the amygdala as well as the hippocampus, and consequently alleviated the impaired extinction of contextual fear.

On the other hand, chronic pre-exposure of DCS has been reported to eliminate effects upon fear extinction (Parnas *et al*, 2005). Similarly, Quartermain et al. (1994) reported that chronic pre-exposure to DCS for 15 days led to a significant decrease in the effect of DCS upon the maze learning test (Quartermain *et al*, 1994). The discrepancy observed in the DCS effect upon extinction may be due to differences in experimental procedures. Studies reported by Parnas et al. (2005) and Quartermain et al. (1994) described the multiple administration of DCS before fear conditioning or the maze learning test, respectively (i.e., pre-administration of DCS). In contrast, in the present study, we administered DCS after fear conditioning but with extinction training. Therefore, it is conceivable that tachyphylaxis or the desensitization of NMDA receptors, as proposed by Parnas et al. (2005), may not have occurred in our study.

As mentioned above, the initial level of freezing is also critical in the assessment of contextual fear extinction by DCS. To our knowledge, little has been reported previously concerning the effects of DCS upon fear consolidation. Therefore, in the present study, we

examined the possibility that DCS affected the consolidation of original fear. Although we administered DCS after fear conditioning, the results of our study demonstrate that DCS did not affect the consolidation of fear 24 hr after fear conditioning. Subsequently, DCS was found to enhance extinction, rather than consolidation of fear. It is too early to conclude the precise effects of DCS upon fear consolidation from this result; further studies using different experimental paradigms are required.

Studies investigating the clinical use of DCS in the treatment of acrophobia or social anxiety disorder have demonstrated that the acute dosing of DCS enhances fear extinction (Hofmann *et al*, 2006; Ressler *et al*, 2004). On the other hand, studies reported by Heresco-Levy et al. (2002) did not demonstrate sufficient efficacy for DCS in the treatment of PTSD (Heresco-Levy et al, 2002). This discrepancy could be attributed to procedural difference. Heresco-Levy et al. (2002) used daily chronic dosing without exposure therapy whilst Hofmann et al. (2006) and Ressler et al. (2004) used acute dosing with exposure therapy. This, considered collectively with findings from animal studies, leads to the postulation that DCS administration without extinction training cannot facilitate fear extinction.

In Experiment 3, our analyses of NMDAR expression in the hippocampus demonstrated that the levels of all 4 NMDAR subunits in SPS rats were significantly higher than those in

Sham rats before and during the entire period of extinction training. Additionally, in Experiment 4, we also found that the administration of DCS eliminated the enhanced mRNA levels of NR2A and NR2B in SPS rats. It is difficult to explain these results. However, one possible explanation could be that SPS leads to NMDA hypofunction in the hippocampus thereby causing a homeostatic increase in NMDA receptor expression. This would account for the relative reduction in neural plasticity. However, the mechanism of action for DCS would actually be more complicated. DCS, irrespective of its mechanism of involvement in the enhancement of fear extinction, may help to reverse hippocampal plasticity, and thus reverse NMDA compensatory alterations.

Recent studies have indicated that hippocampal neural plasticity is involved in fear extinction (Corcoran *et al*, 2005; Fischer *et al*, 2004; Heldt *et al*, 2007). For example, Corcoran et al. (2005) have demonstrated that muscimol inactivation of the dorsal hippocampus reduced the rate of extinction and prevented the context dependency of extinction. Previous studies by this group indicated that treating the dorsal hippocampus with muscimol produced selective impairment in the context specificity of extinction (Corcoran and Maren, 2001). In addition, Fischer et al. (2004) have shown that structural plasticity involving actin rearrangement within dorsal hippocampus is required for contextual fear extinction. More recently, Heldt et al. (2007) demonstrated that the hippocampal expression

of BDNF is required for the neural plasticity underlying the acquisition or consolidation of extinction memories.

In our study, we did not clarify the mechanism by which DCS alleviated the impairment of extinction in SPS rats. Although the precise mechanism underlying how DCS facilitates fear extinction remains to be determined, several studies have suggested that DCS activates NMDAR-mediated signal transduction, thus contributing to the enhancement of new learning (Gabriele and Packard, 2007; Land and Riccio, 1999; Quartermain *et al*, 1994; Rouaud and Billard, 2003). For example, Rouaud and Billard (2003) showed that DCS facilitated NMDAR-mediated signal transduction and synaptic plasticity in the CA1 field of rat hippocampal slices. Interestingly, Davis and colleagues have recently proposed that the NMDARs involved in extinction may be different from those involved in fear conditioning (e.g., on different neurons) (Davis *et al*, 2006). To test this hypothesis, further research is warranted.

The limitations of the present study are as follows. Firstly, we administered DCS to the rats orally in an effort to avoid stress incurred via injection and to mimic clinical use. However, using this methodology it was not possible to accurately confirm the amount of Milli-Q water consumed by each rat daily. Hence, it should be noted that the concentration of DCS can only be assumed to be approximately 15mg/kg. Secondly, we did not evaluate

changes in the protein level of NMDARs, the extent of NMDAR phosphorylation, or intracellular signal transduction mediated by NMDARs in the hippocampus. Converging evidence has indicated that the phosphorylation of mitogen-activated kinase (MAPK)/extracellular signal-regulated kinase (ERK) in the hippocampus via NMDARs plays an important role in fear extinction (Fischer *et al*, 2007; Szapiro *et al*, 2003). Therefore, additional studies using immunoblot or immunohistochemical analyses are necessary to address these issues. Thirdly, it should be noted that because there is enhanced NMDA receptor expression in the hippocampus, this does not mean that the same is happening in other areas of brain. In addition, it is plausible that the enhanced NMDA receptor expression observed in the hippocampus could be a consequence, but not a causative factor, of impaired fear extinction. Finally, we cannot rule out the possibility of the involvement of other brain regions in fear extinction as described above. Numerous studies have suggested that the amygdala and medial prefrontal cortex (mPFC) also play an important role in extinction (Barad *et al*, 2006; Quirk *et al*, 2006; Sotres-Bayon *et al*, 2006). The hippocampus has strong reciprocal connections with the mPFC and the amygdala; these three brain regions seem to interactively coordinate the encoding and expression of fear extinction (Sotres-Bayon *et al*, 2006).

In summary, SPS rats showed impaired fear extinction in the contextual fear conditioning

paradigm. Furthermore, repeated DCS administration ameliorated the impaired extinction in SPS rats. This is the first study indicating the efficacy of DCS upon fear extinction in a conditioned stress model of PTSD. Although the precise mechanism of fear extinction remains unknown, the results of the present study indicate that NMDAR-mediated signal transduction in the hippocampus may be involved in the pathophysiology of fear extinction. Studies concerning fear extinction using the intra-hippocampus infusion of DCS have yet to be performed. Further studies administering DCS into the hippocampus would greatly enhance our understanding of the mechanisms underlying DCS action. Further studies examining both the neural mechanisms underlying the effect of DCS and the efficacy of DCS in the treatment of PTSD are needed to further our understanding of the pathophysiology of PTSD and for the development of novel therapeutic strategies for PTSD.

Supplementary information is available at the *Neuropsychopharmacology* website.

Disclosure/Conflict of Interest

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Figure legends

Fig. 1 Treatment groups and procedure. In the SPS group, 7 days after SPS treatment, contextual fear conditioning was performed (on day 0), and then extinction training was performed on each of 5 consecutive days following fear conditioning. In the Sham group, the procedures were similar except that SPS treatment was not performed.

Fig. 2 Influence of SPS on fear extinction. Data are expressed as mean ± SEM of 10 rats per group. On day 1, there were no significant differences in the freezing levels between the Sham and SPS groups. However, on days 2 to 5, the SPS group showed impaired extinction compared to the Sham group. *P ＜ 0.05, **P < 0.01; unpaired student's t test.

Fig. 3 Effects of repeated DCS administration on fear extinction. Data are expressed as mean ± SEM of 10 rats per group.

Fig. 4 Effects of repeated DCS administration upon fear extinction a week after extinction training. Data are expressed as mean ± SEM of 9 rats per group.

Fig. 5 Comparison of the levels of NMDAR mRNAs [NR1 **(A)**, NR2A **(B)**, NR2B **(C)**, NR2C

(D)] across experimental timepoints. Data are expressed as the ratio of the concentration of the target molecule to that of GAPDH (target molecule/GAPDH) and represent the mean ± SEM (day 0: Sham; N = 6, SPS; N = 6, day 1: Sham; N = 7, SPS; N = 7, day 4: Sham; N = 9, SPS; N = 9).

Fig. 6 Effects of repeated DCS administration on the levels of NMDAR mRNAs [NR1 **(A)**, NR2A **(B)**, NR2B **(C)**, NR2C **(D)**] on day 4. Data are expressed as the ratio of the concentration of the target molecule to that of GAPDH (target molecule/GAPDH) and represent the mean ± SEM of 9 rats per group.

Fig. 1

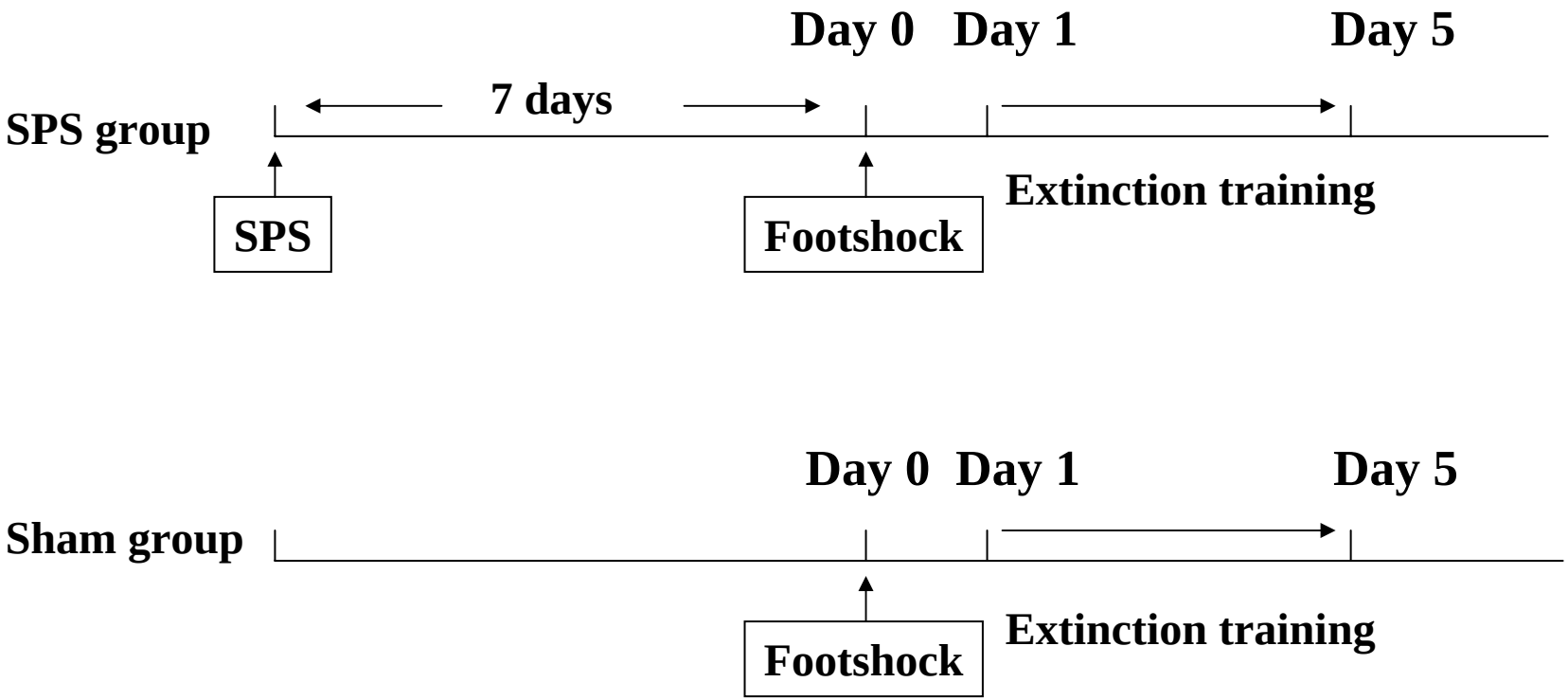

Day 0
Day 1
Day 5
7 days
SPS group
SPS
Footshock
Extinction training
Day 0
Day 1
Day 5
Sham group
Footshock
Extinction training

Fig. 2

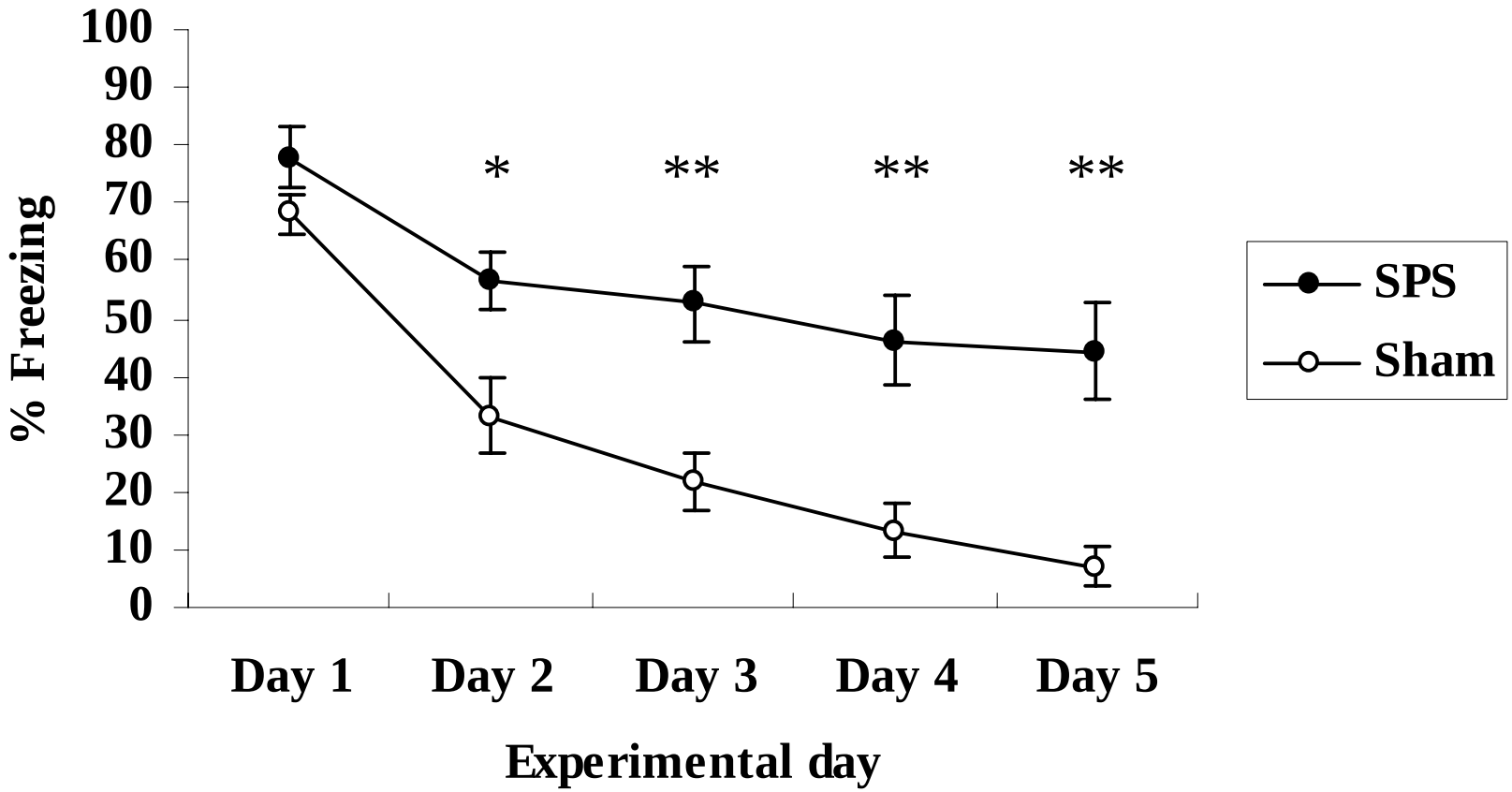

100
90
80
70
60
50
40
30
20
10
0
% Freezing
*
**
**
**
Day 1
Day 2
Day 3
Day 4
Day 5
Experimental day
SPS
Sham

Fig. 3

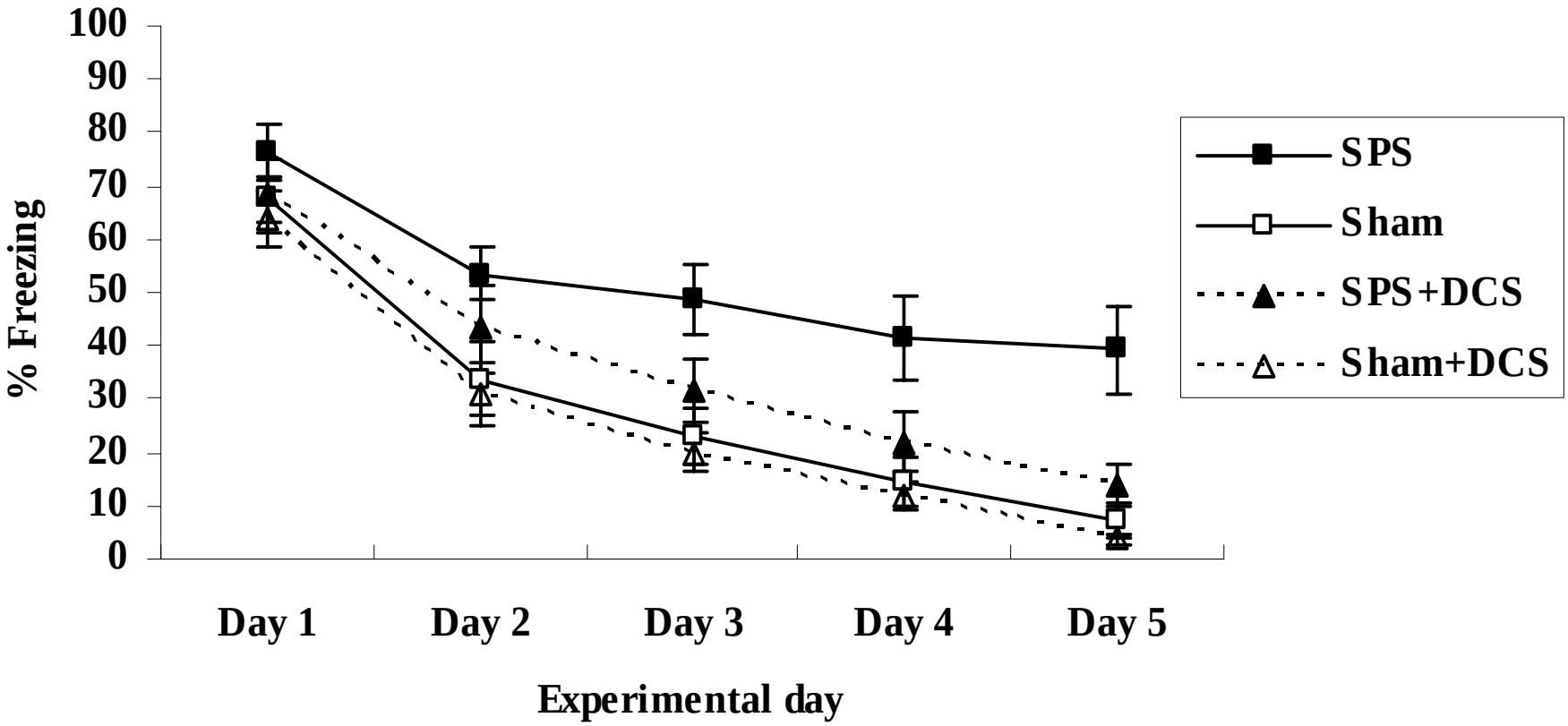

100
90
80
70
60
50
40
30
20
10
0
% Freezing
Day 1
Day 2
Day 3
Day 4
Day 5
Experimental day
SPS
Sham
SPS+DCS
Sham+DCS

Fig. 4

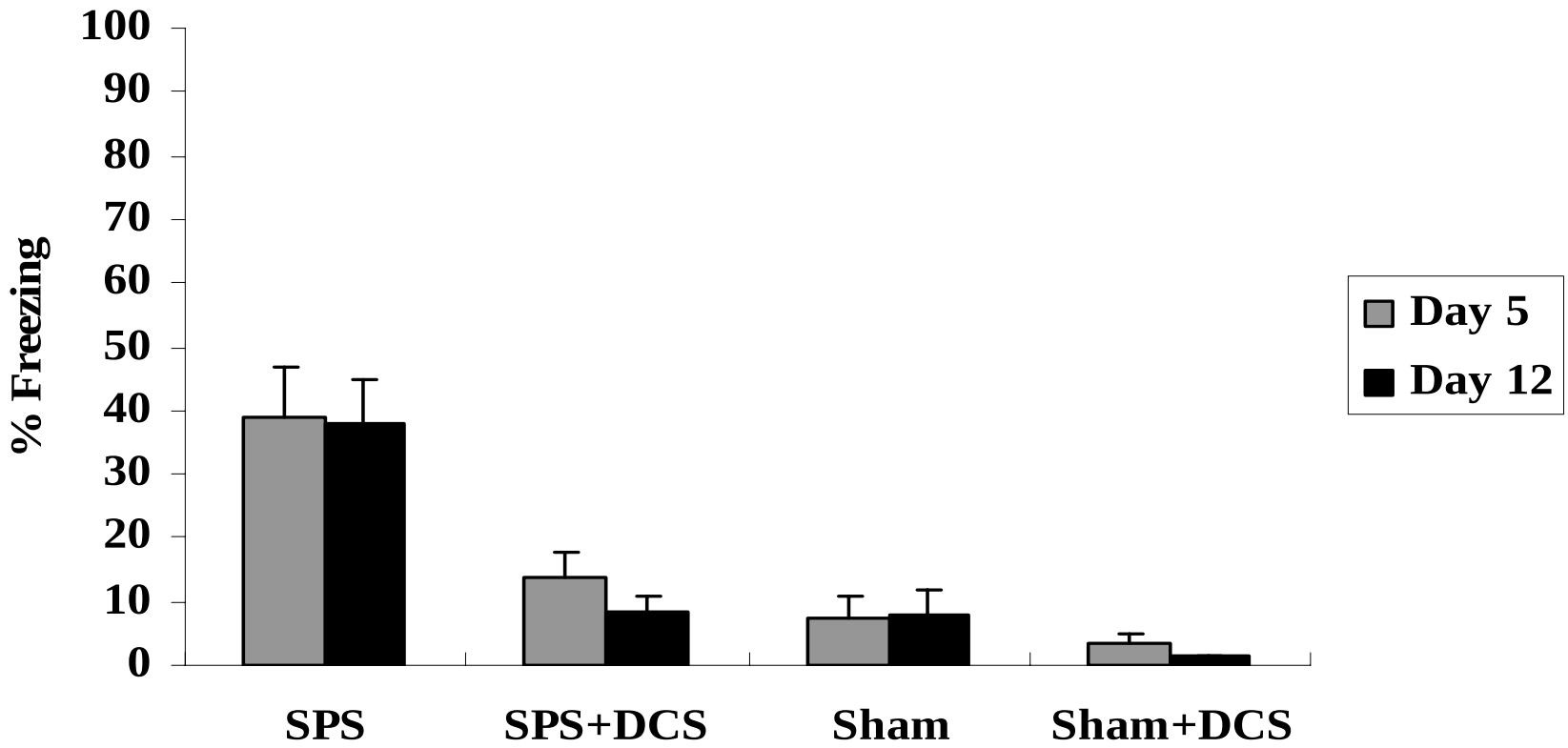

100
90
80
70
60
50
40
30
20
10
0
% Freezing
Day 5
Day 12
SPS
SPS+DCS
Sham
Sham+DCS

Fig. 5A

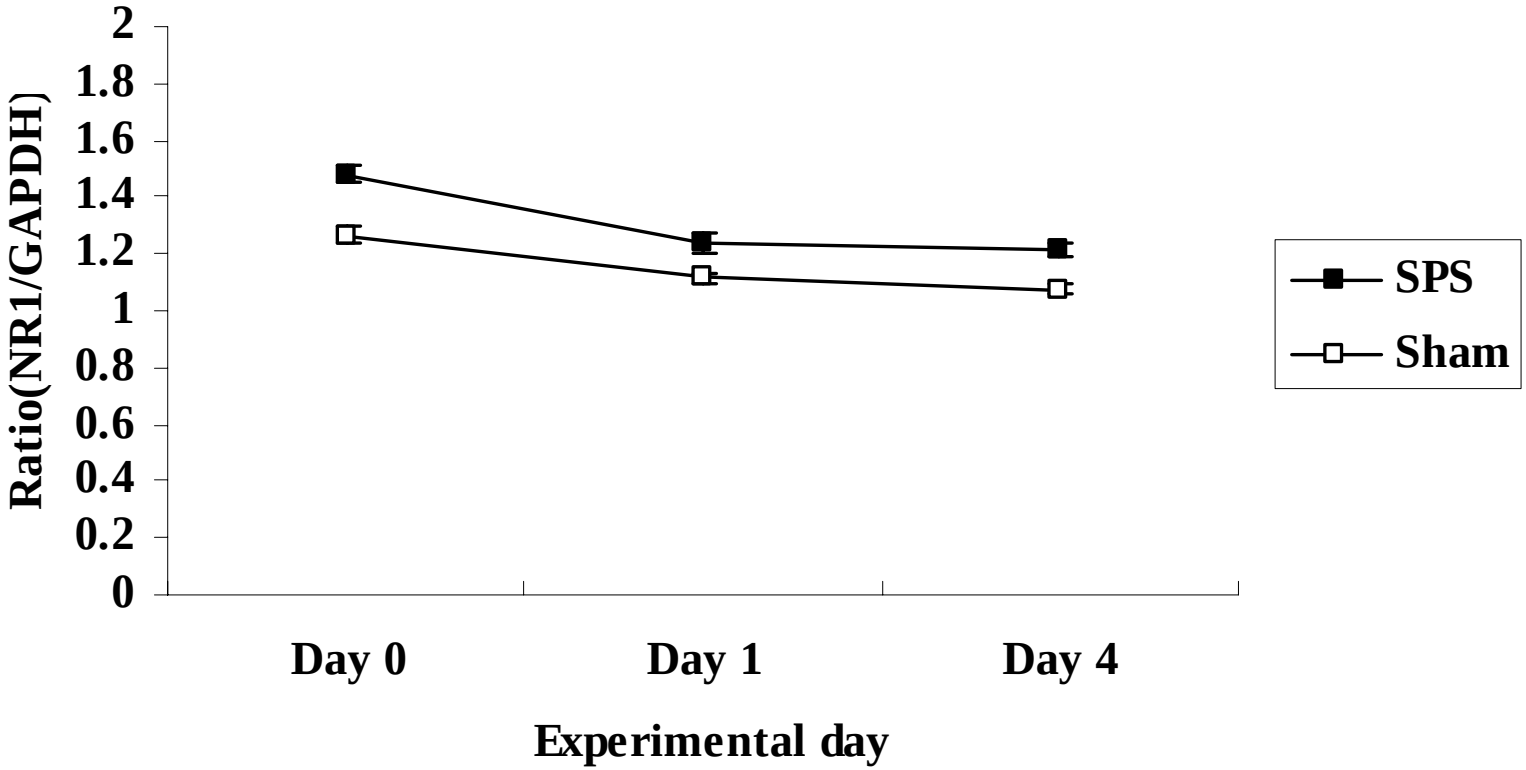

Ratio(NR1/GAPDH)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Day 0
Day 1
Day 4
Experimental day
SPS
Sham

Fig. 5B

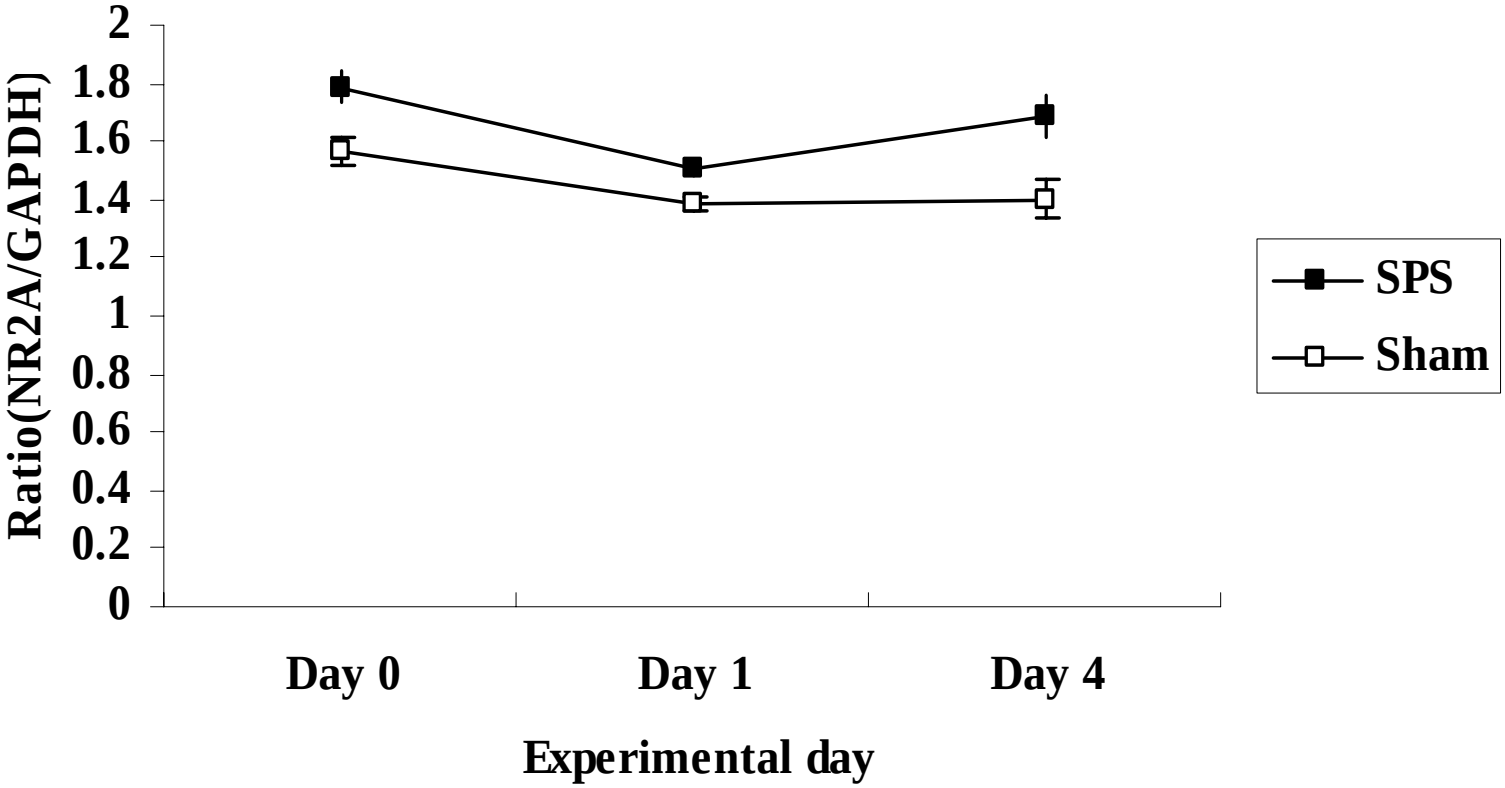

Ratio(NR2A/GAPDH)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Day 0
Day 1
Day 4
Experimental day
SPS
Sham

Fig. 5C

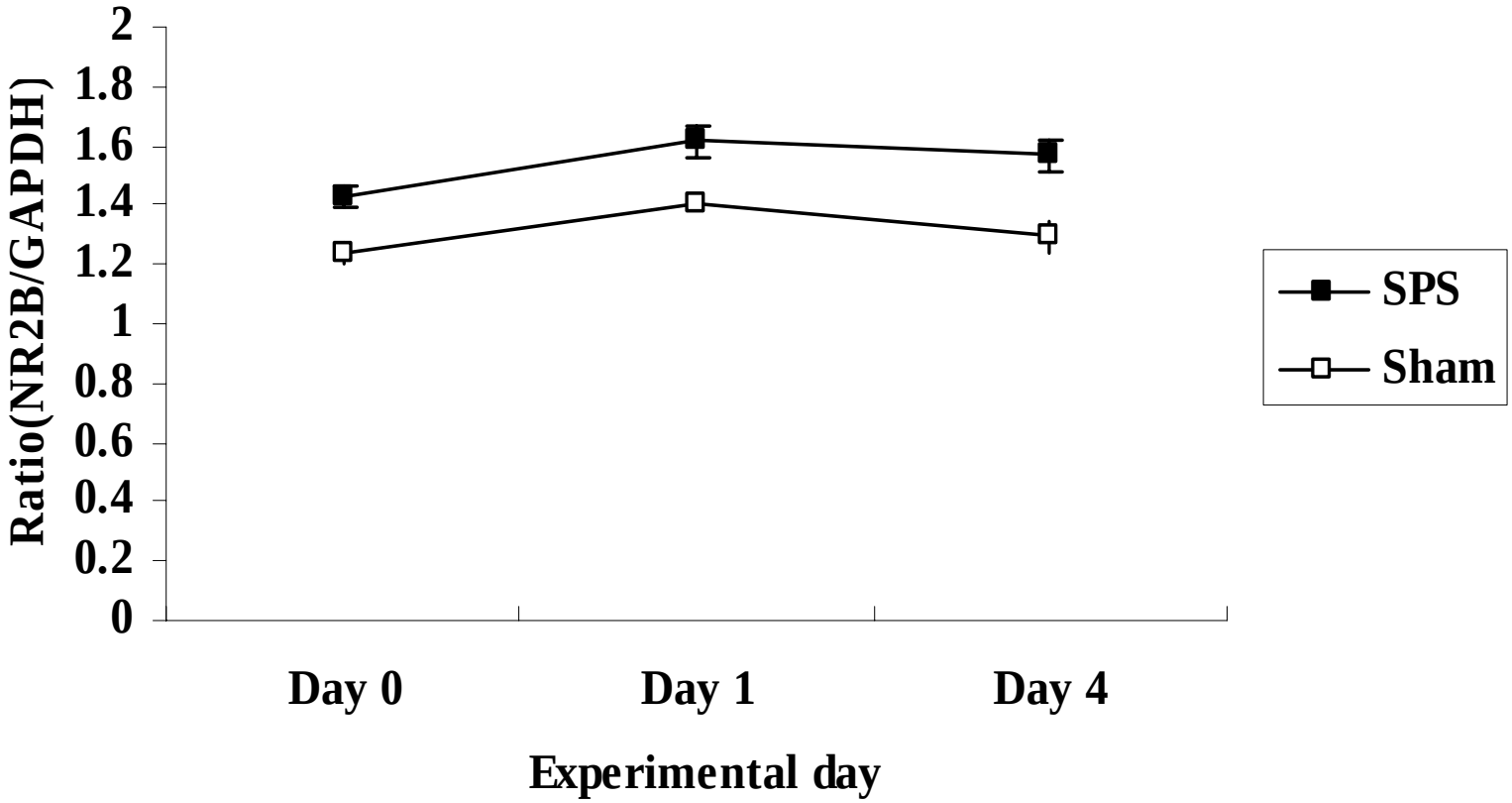

Ratio(NR2B/GAPDH)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Day 0
Day 1
Day 4
Experimental day
SPS
Sham

Fig. 5D

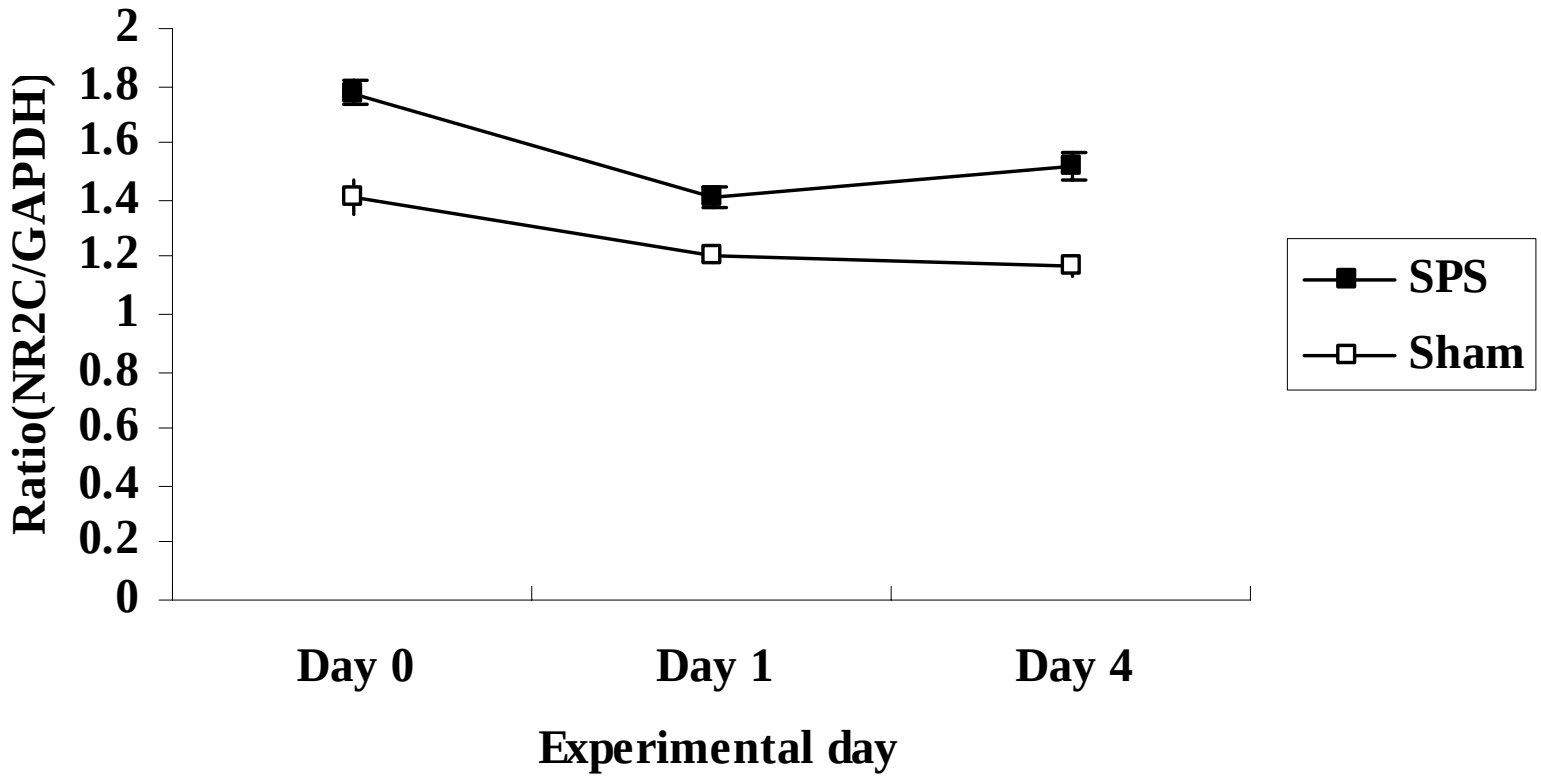

Ratio(NR2C/GAPDH)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Day 0
Day 1
Day 4
Experimental day
SPS
Sham

Fig. 6A

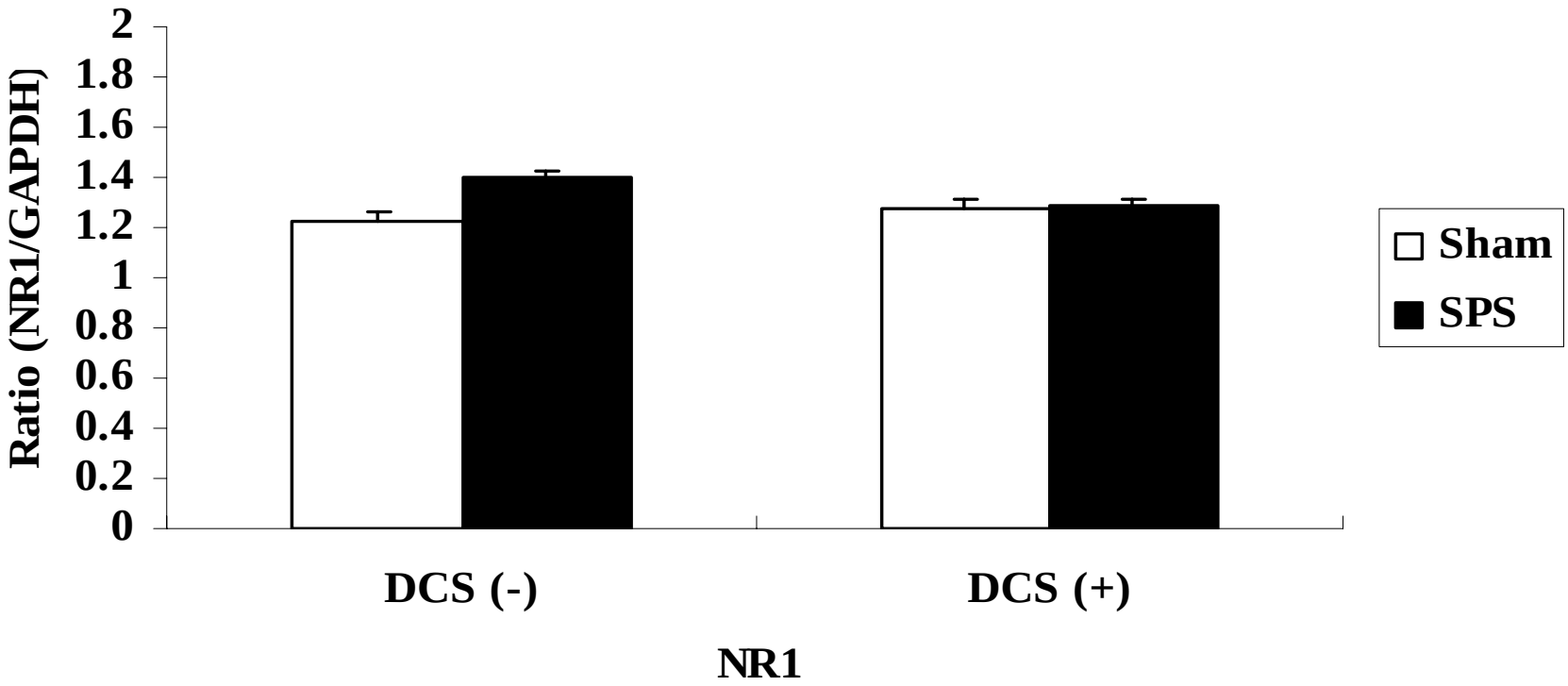


Fig. 6B

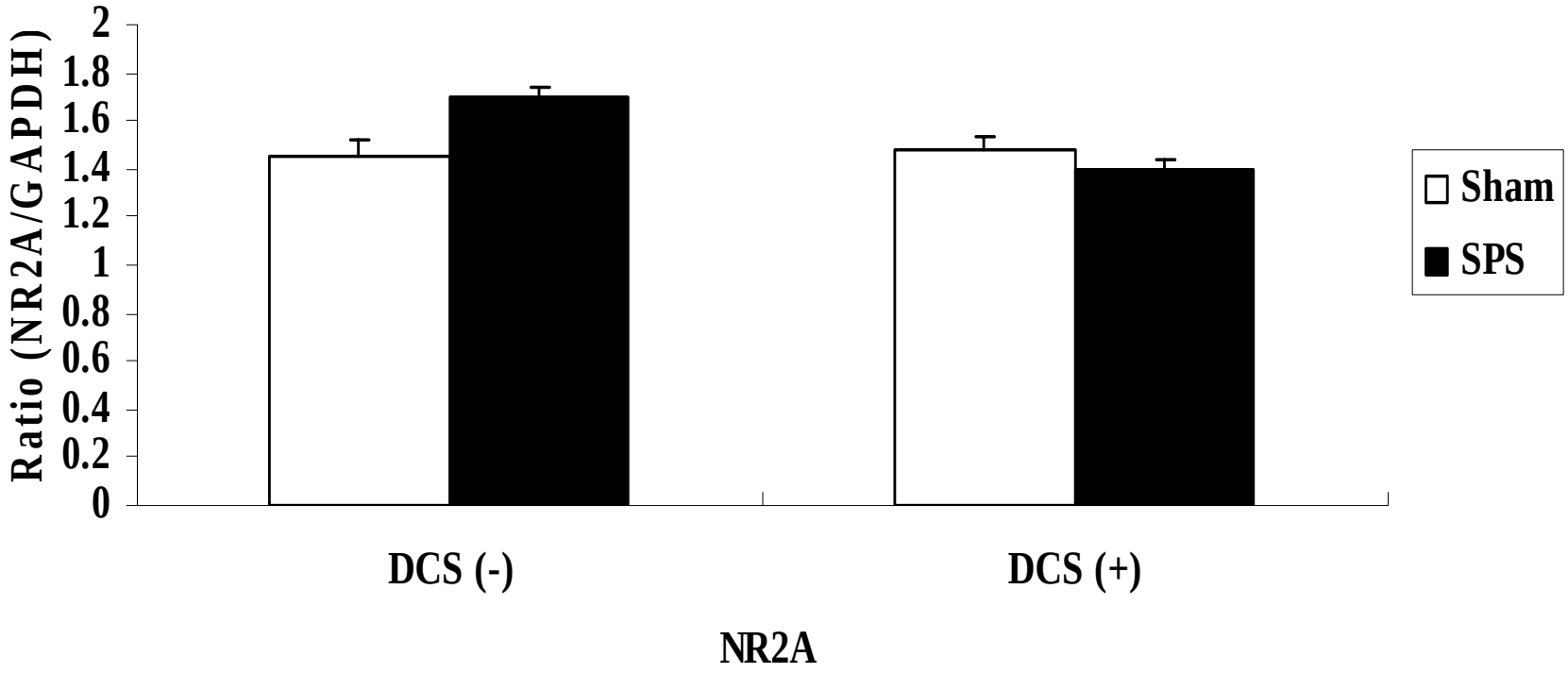

Ratio (NR2A/GAPDH)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
DCS (-)
DCS (+)
NR2A
Sham
SPS

Fig. 6C

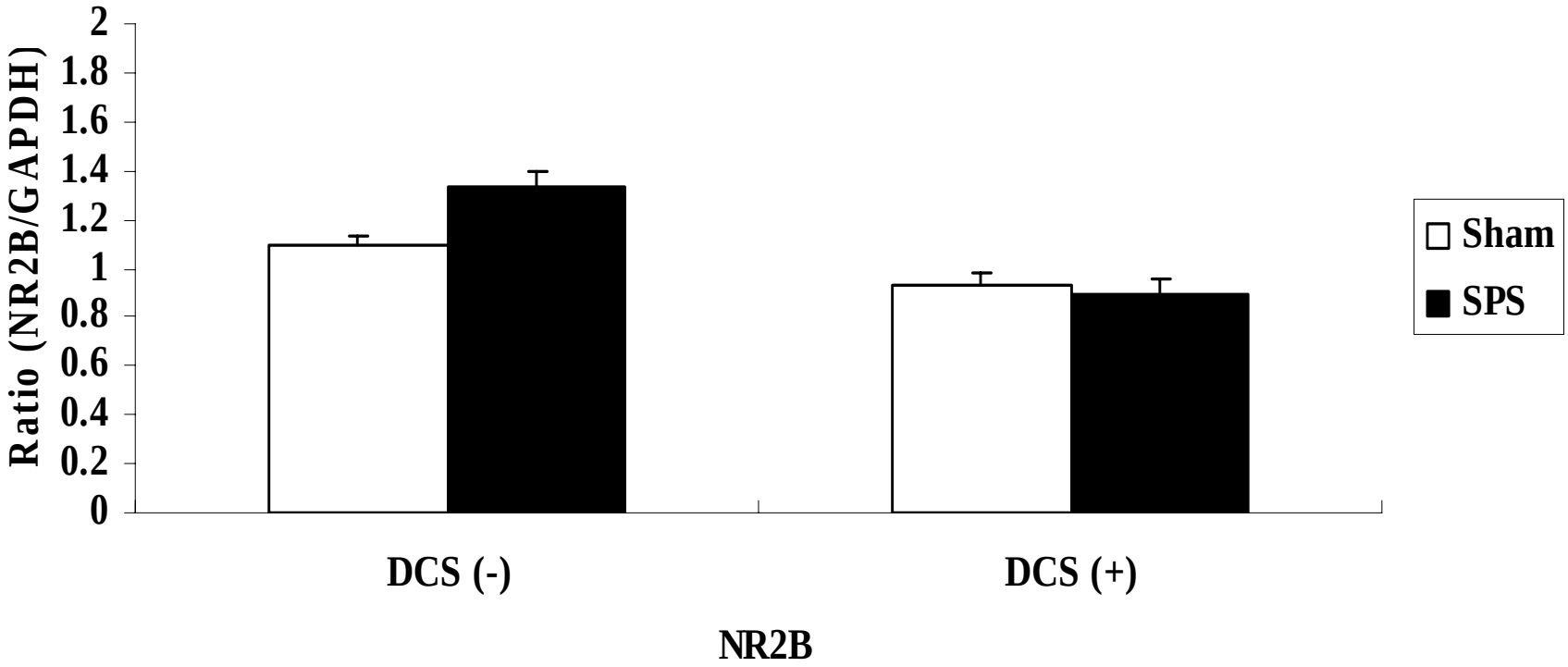
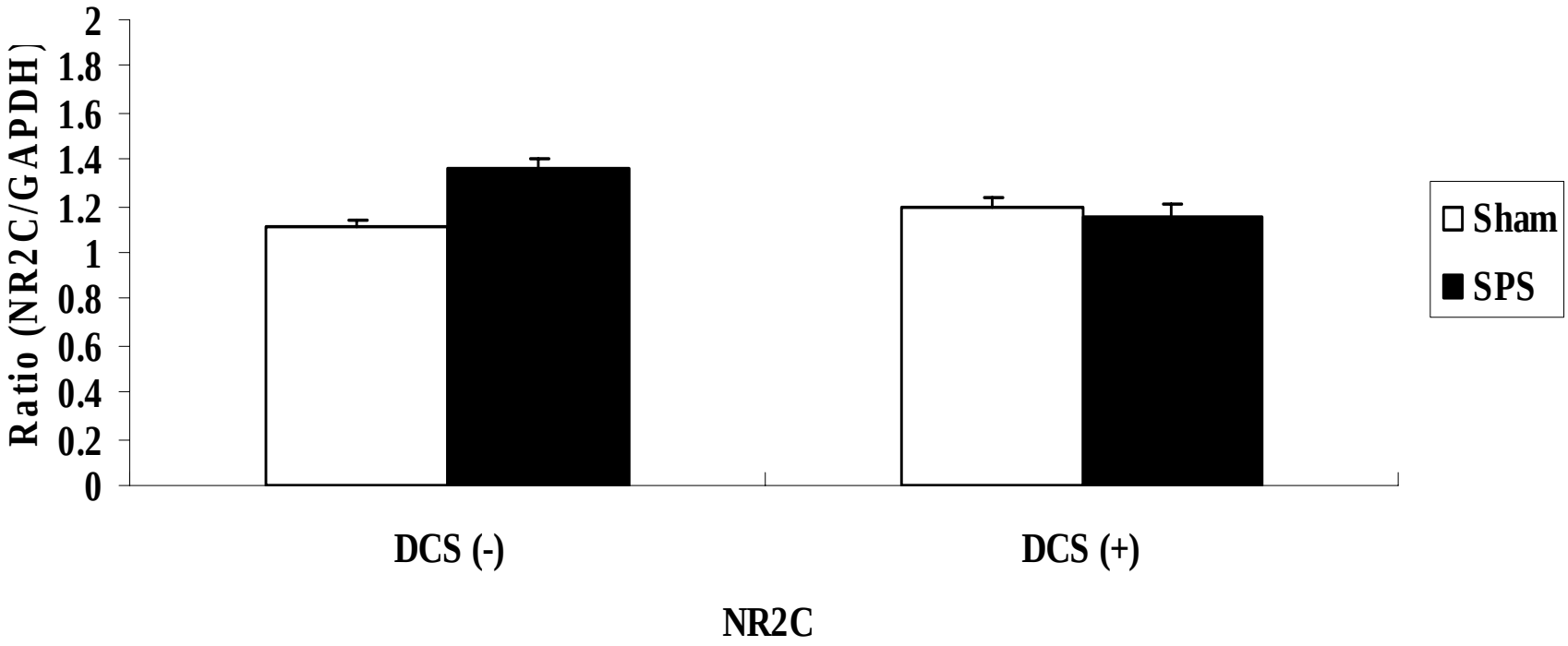

Ratio (NR2B/GAPDH)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
DCS (-)
DCS (+)
NR2B
Sham
SPS

Fig. 6D


Ratio (NR2C/GAPDH)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
DCS (-)
DCS (+)
NR2C
Sham
SPS

Supplementary Fig. 1

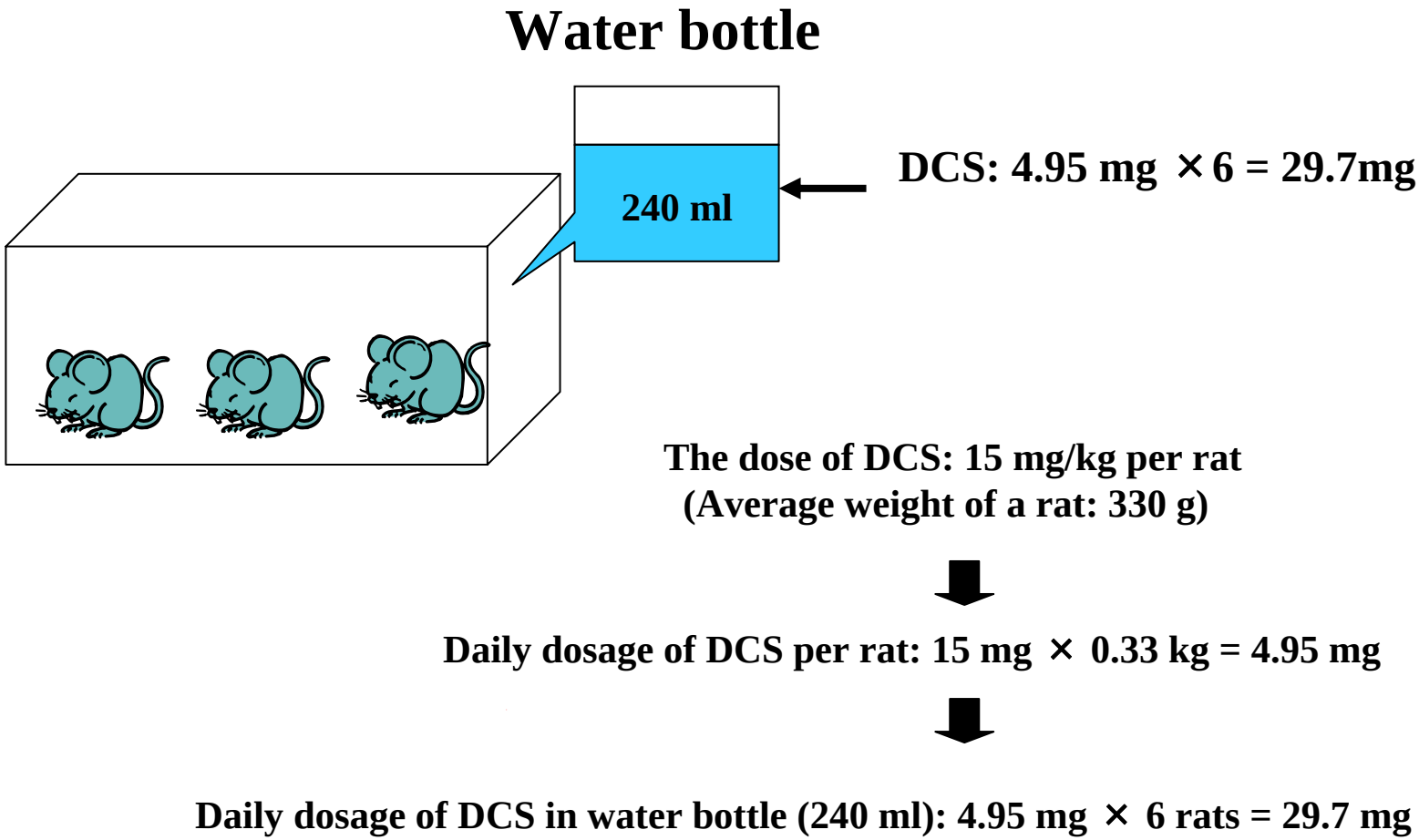


Fig. s1. DCS administration protocol

Supplementary Fig. 2

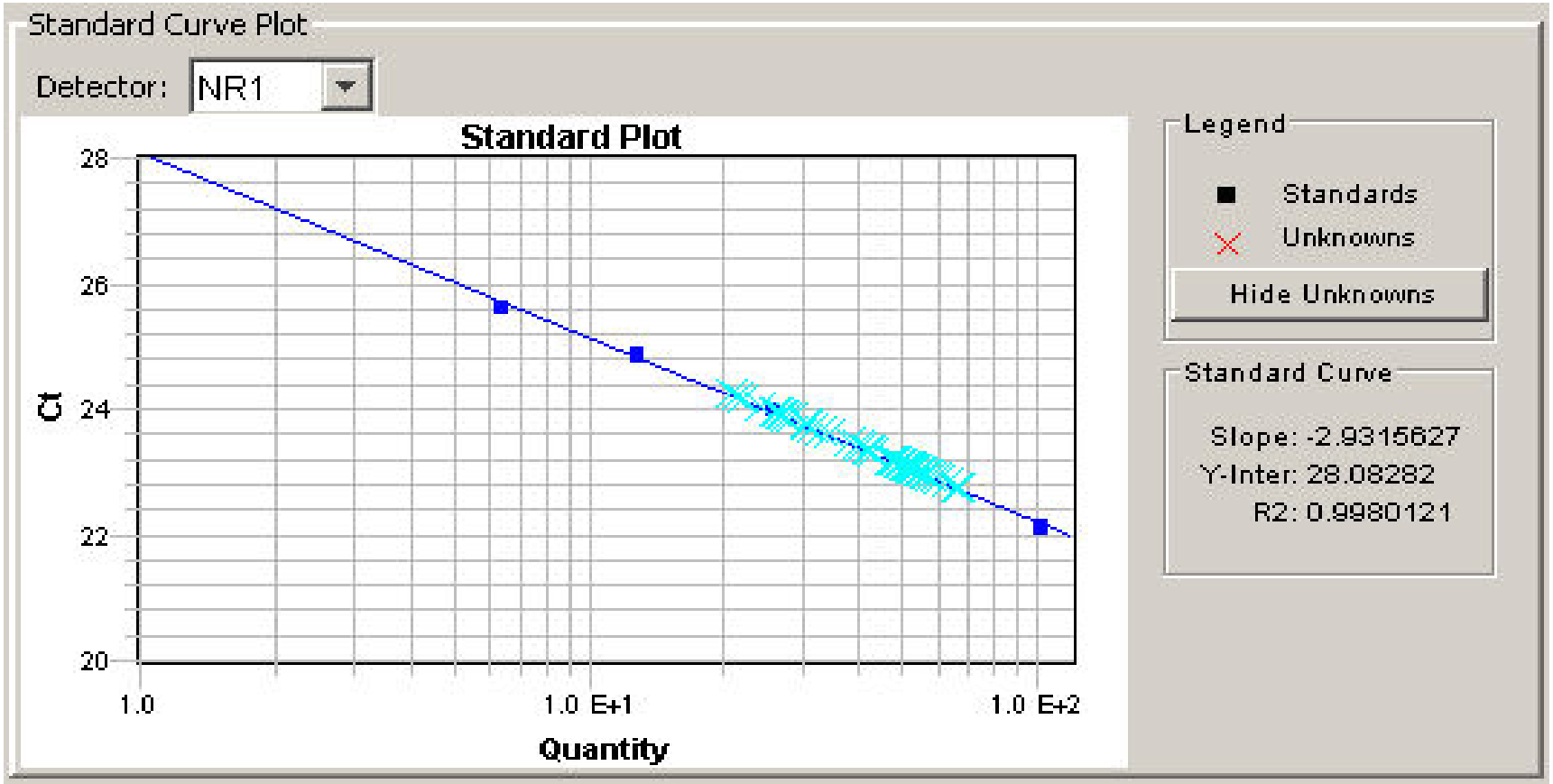


Fig. s2. The standard curve plot displays the samples before fear conditioning on a graph of CT (threshold cycle) vs. Quantity (Log N)

Supplementary Fig. 3

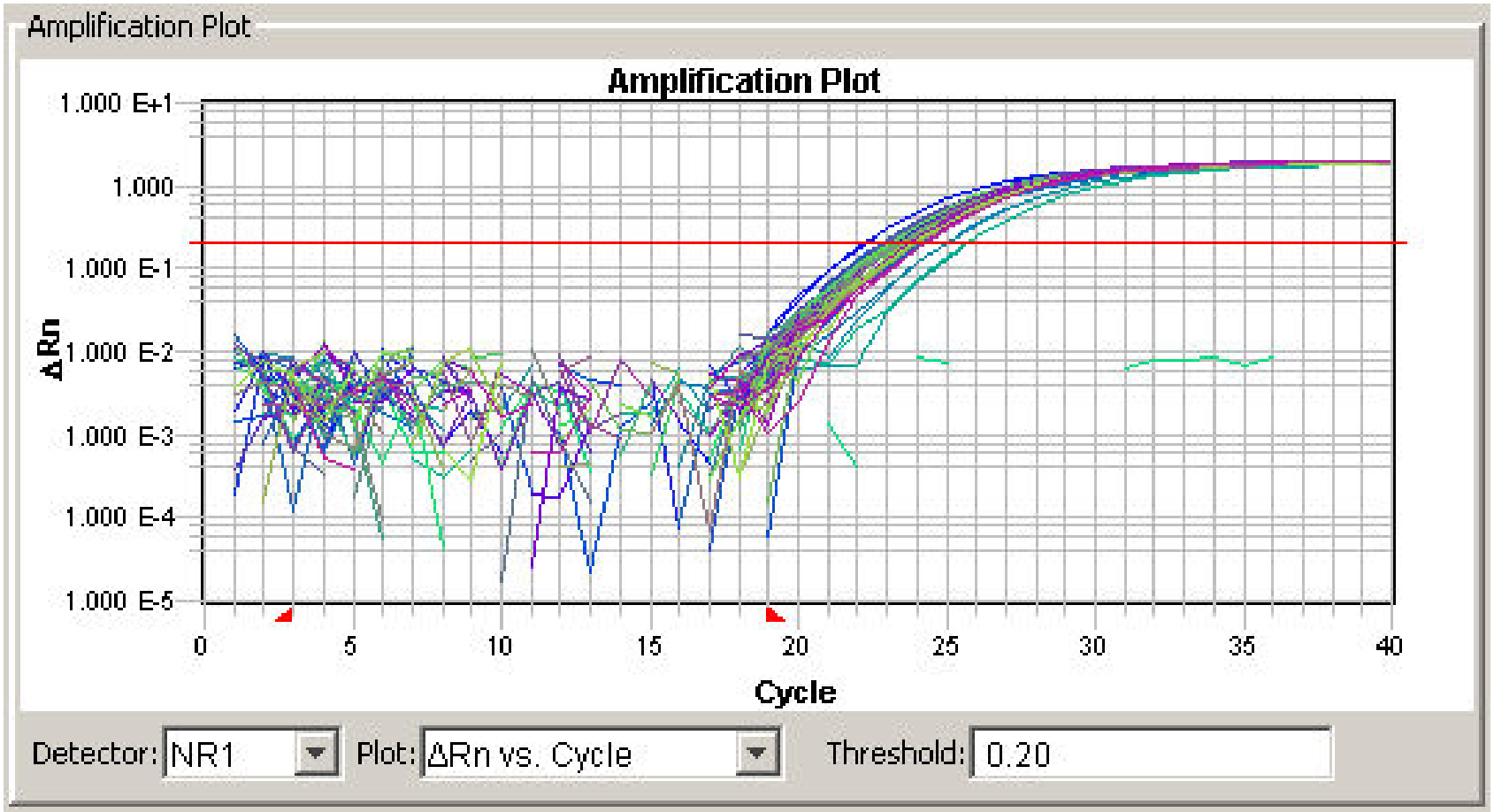


Fig. s3. The amplification curve shows normal amplification of PCR product (NR1).