Alterations in the Hippocampal Glycinergic System in an Animal Model of Posttraumatic Stress Disorder

Shigeto Yamamoto [a], Shigeru Morinobu [a *], Yasuyuki Iwamoto [a], Yuto Ueda [b], Shiro Takei [a], Yosuke Fujita [a], Shigeto Yamawaki [a]

a) Department of Psychiatry and Neurosciences, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima, 734-8551, Japan.

b) Section of Psychiatry, Department of Clinical Neuroscience, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki, 889-1692, Japan.

*** Corresponding author**

Shigeru Morinobu M.D., Ph.D.

Department of psychiatry and Neurosciences, Division of Frontier Medical Science, Programs for biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University.

1-2-3 Kasumi, Minami-ku, 734-8551, Hiroshima, Japan

Tel: +81-82-257-5205, Fax: +81-82-257-5209

E-mail address: smoriob@hiroshima-u.ac.jp

Abstract

Previous studies have demonstrated that rats subjected to single prolonged stress (SPS) exhibit posttraumatic stress disorder (PTSD)-like symptoms, such as enhanced contextual fear in response to trauma-related and trauma-unrelated events. Furthermore, we previously reported that upregulation of hippocampal glycine transporter 1 (GlyT-1) mRNA after context exposure could be the initial mechanism underlying impaired fear extinction in SPS rats. To clarify the involvement of the hippocampal glycinergic system in impaired fear extinction in SPS rats, we measured the time course of changes in the duration of freezing and the hippocampal levels of Gly-T1 mRNA using contextual fear conditioning (FC) and extinction training. We also used in vivo microdialysis to measure the concentration of extracellular glycine in the hippocampus during the time interval between FC and the first context exposure. SPS rats exhibited increased and sustained contextual fear responses. The enhanced contextual fear response in SPS rats was associated with a sustained increase in hippocampal levels of Gly-T1 mRNA after FC relative to sham rats, and by a decrease in the extracellular glycine concentration. GlyT-1 mRNA levels in rats that underwent repeated extinction training were significantly lower than in rats that did not undergo extinction training. These findings indicate that reduced activity of the hippocampal glycinergic system

could be closely involved in impaired fear extinction in SPS rats, suggesting that activation of the glycinergic system by D-cycloserine or GlyT-1 inhibitors may ameliorate the impairment of fear extinction.

Keywords: single prolonged stress, fear extinction, glycine, glycine transporter 1, hippocampus, posttraumatic stress disorder.

1. Introduction

Cognitive behavioral therapy (CBT) is the most commonly used approach for the treatment of posttraumatic stress disorder (PTSD), and its clinical efficacy has been well established (Mendes et al., 2008). Extinction learning, the diminishment of fear evoked by context, plays an important role in the treatment of PTSD. In fact, one of the main clinical characteristics of PTSD is exaggerated and persistent fear responses to reminders of the traumatic event, and CBT relies on extinction-based mechanisms (Rothbaum and Davis, 2003). Based on these findings, it is hypothesized that impaired fear extinction may be associated with the pathophysiology of PTSD.

D-cycloserine (DCS), a partial agonist at the N-methyl-D-aspartate receptor (NMDAR), is considered to be a promising pharmacological agent for the treatment of PTSD, because DCS has been shown to facilitate extinction learning in rodent studies (Ledgerwood et al., 2004, 2005; Walker et al., 2002) and in human trials of anxiety disorders, such as acrophobia (Ressler et al., 2004) and social anxiety disorder (Guastella et al., 2008; Hofmann et al., 2006), and obsessive compulsive disorder (Kushner et al., 2007). Several studies are currently evaluating the use of DCS to enhance imaginal exposure or virtual reality exposure therapy for the treatment of PTSD; however, this research is still in the preliminary stages (Cukor et al., 2009).

Yamamoto et al. (2008) recently reported that rats subjected to single prolonged stress (SPS), which is an animal model of PTSD first proposed by Liberzon et al. (1997, 1999), exhibited impaired fear extinction relative to rats not subjected to SPS (sham rats). The study also showed that DCS administration with extinction training ameliorated the impairment of fear extinction in SPS rats (Yamamoto et al., 2008). However, the precise mechanism by which co-administration of DCS reduces fear evoked by the traumatic context remains unknown.

In a different fear conditioning paradigm, Iwamoto et al. (2007) demonstrated that 24 h after contextual fear conditioning (FC), SPS rats exhibited a significant increase in contextual freezing as compared with sham rats (Iwamoto et al., 2007). That study also suggested that up-regulation of glycine transporter 1 (GlyT-1) in the hippocampus after reexposure to the context would be the initial event in the development of impaired extinction in SPS rats.

GlyT-1 plays an important role in modulating extracellular glycine concentrations, and glycine serves to modulate NMDAR function via the glycine-B binding site on the NR1 subunit of the NMDAR. In support of these mechanisms, several studies have reported that inhibition of GlyT-1 activity increases extracellular glycine availability in the CNS and can enhance neurotransmission via NMDARs (Sur and Kinney, 2007).

In the present study, the SPS paradigm was used to clarify the mechanisms underlying fear extinction in relation to the hippocampal glycinergic system. First, we examined contextual fear responses at two time-points after fear conditioning. Second, we measured the time course of changes in the levels of Gly-T1 mRNA by real-time quantitative polymerase chain reaction (RT-PCR). In addition, we examined whether a correlation existed between hippocampal Gly-T1 mRNA level and fear responses due to repeated context exposure (i.e. extinction training). Lastly, we measured the extracellular glycine concentration in the hippocampus by in vivo microdialysis at the time of FC and of exposure to the context alone.

2. Materials and Methods

2.1. Animals

Male Sprague-Dawley rats weighing between 300 g and 350 g (Charles River Japan, Yokohama, Japan) were used in the 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 different set of rats was used for each of the methods (i.e., contextual fear test, RT-PCR, and in vivo microdialysis). All animal procedures were conducted in strict accordance with the Hiroshima University

School of Medicine Animal Care Committee's Guiding Principles on Animal Experimentations in Research Facilities for Laboratory Animal Science.

2.2. 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 h, forced swim for 20 min, and ether anesthesia. Each rat was restrained for 2 h by placing it inside a disposable clear polyethylene cone bag (Asahikasei, Tokyo, Japan) with only the tail protruding. 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, the rats were individually placed in a clear acrylic cylinder (240 mm D ×500 mm H), 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 cages for 7 days.

2.3. Contextual fear conditioning (FC) and context exposure (CE)

In the first experiment, we investigated the influence of SPS on contextual fear

(Experiment 1). Animals were randomly assigned to two groups (SPS or sham). Sham rats were left alone in their cages without handling. Rats were placed in a conditioning chamber (325W×280H×500D mm), and then were exposed to a 180-sec conditioning context without any stimulation (i.e., a tone). Immediately afterwards, 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). Following the footshock, rats remained in the chamber for an additional 1 min before being returned to their home cages.

Twenty-four hours after FC, rats were placed for 3 min without footshock in the same chamber where the footshock was delivered. In this manner, context exposure (CE) was performed once daily for 2 days (Fig. 1). Additionally, in the SPS group, CE was continued for up to 7 days, to clarify the relation between contextual fear and the GlyT-1 mRNA expression (Experiment 2b). In this experiment, SPS rats without extinction training were used as controls (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 was recorded on videotape and later scored

blindly by well-trained experimenters. Pearson's correlation coefficient was calculated to determine inter-rater reliability between the two scorers, which was high (r = 0.96).

2.4. Real-time quantitative polymerase chain reaction (RT-PCR)

To examine the involvement of the hippocampal glycinergic system in contextual fear, we used RT-PCR to measure alterations in GlyT-1 mRNA levels in the hippocampus (Experiment 2a). Animals were randomly assigned to two groups (SPS, sham) and were sacrificed by decapitation at the indicated time points: before FC, immediately after FC, before the first CE, immediately after the first CE (Fig. 1). In Experiment 2b, animals were sacrificed by decapitation immediately after CE on day 8 (Fig. 1). Hippocampal tissue was removed from the brain, 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 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. GlyT-1 mRNA was amplified by RT-PCR. 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 probe for GlyT-1. 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. 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 at 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). GlyT-1 mRNA levels 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 in unknown samples was calculated.

2.5. In vivo microdialysis

We performed in vivo dialysis in the ventral hippocampus to measure the extracellular glycine concentration using HPLC with electrochemical detection (HPLC-ECD) (Experiment 3). Animals were randomly assigned to two groups (SPS and

sham). Dialysates were collected at 25 min intervals before and after the footshock stimulation until 125 min post-stimulus. Similarly, 24 h after the footshock, dialysates were also collected at 25 min intervals before and after the first CE until 125 min post-stimulus. Dialysis samples were stored at -80°C until analysis by HPLC-ECD. Under sodium pentobarbital anesthesia (40 mg/kg), each rat underwent stereotaxic implantation of a guide cannula into the ventral hippocampus. The stereotaxic coordinates were determined according to the atlas of Paxinos and Watson (Paxinos and Watson, 1986), and the incisor bar was set at the intraaural line. A 22-G guide cannula was implanted stereotaxically in each rat so that the tip was 5.0 mm posterior and 5.5 mm to the right of bregma and 3.0 mm below the surface of the skull. The cannula was firmly anchored to the skull with screws and dental cement. A microdialysis probe was prepared for insertion into the guide cannula by covering the probe's tip with a 4.0-mm length of semi-permeable hollow fiber (11 μm thick, 0.22 mm outside diameter, molecular weight cut-off 50,000 Da; regenerated cellulose Eicom Corporation, Japan). Basal concentrations of glycine in the ventral hippocampus were measured using HPLC-ECD. Prior to the HPLC-ECD analysis, o-phthalaldehyde (OPA) solution was made by adding 13.5 mg of OPA and 10 μl of 2-mercaptoethanol to 2.5 ml of 0.1 M K_2CO_3 buffer (pH 9.6) with 10% ethanol. The sample (30 μl) was mixed with 10 μl of

OPA solution and incubated for 10 min at 4°C. After mixing, 40 μl of the reactant was applied to the HPLC's ODS column. Detection was performed by ECD (Eicom, Tokyo, Japan) at + 600 mV/Ag/AgCl. The elution buffer contained 60 mM $NaH_2PO_4.2H_2O$, 9.6 mM $Na_2PO_4.12H_2O$, 30% methanol, and 0.5 mM EDTA (pH 6.5).

2.6. 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, time) for repeated measures (time). In Experiment 2, data were analyzed by two-way ANOVA (stress, time) or unpaired Student's t test. In Experiment 3, two-way ANOVA (stress, time) was used for repeated measures (time). In Experiments 1-3, post-hoc analysis was conducted using Tukey's test or unpaired Student's t test across time-points, if necessary, to further evaluate the time course change. Results were considered statistically significant at $P < 0.05$.

3. Results

3.1. Experiment 1: The influence of SPS on contextual fear response

In the analysis of freezing responses, two-way ANOVA showed significant main

effects of time [F (1, 14) = 5.34, $p < 0.05$], and stress [F (1, 14) = 14.40, $p < 0.05$]. There was no interaction between time and stress [F (1, 14) = 3.57, $p = 0.07$]. Post hoc analysis revealed that the SPS group showed more freezing responses than the sham group on days 2 and 3 [day 2: t (14) = 2.90, $p < 0.05$; day 3: t (14) = 4.46, $p < 0.05$] (Fig 2).

3.2. Experiment 2a: The levels of GlyT-1 mRNA at several time-points

In the analysis of GlyT-1 mRNA levels, two-way ANOVA showed significant main effects of time [F (3, 40) = 26.96, $p < 0.05$], and stress [F (1, 40) = 2.93, $p < 0.05$]. There was no interaction between time and stress [F (3, 40) = 1.85, $p = 0.15$]. Post hoc analysis revealed no significant differences in GlyT-1 mRNA levels between the SPS and sham groups, before and after FC [before FC: t (10) = 0.96, $p = 0.35$; after FC: t (10) = 1.85, $p = 0.09$]. In contrast, GlyT-1 mRNA levels in the SPS group were significantly higher than those in the sham group, before and after the first CE [before the first CE: t (10) = 5.29, $p < 0.05$; after the first CE: t (10) = 3.38, $p < 0.05$] (Fig. 3).

3.3. Experiment 2b: The relation between contextual fear and the GlyT-1 mRNA expression in the hippocampus

In SPS rats, we first compared the freezing response between the extinction and non-extinction groups on day 8. The freezing response in the extinction group was significantly lower than that in the non-extinction group [$t(14) = 9.95$, $p < 0.05$] (Fig. 4a). Next, we compared the levels of GlyT-1 mRNA between the extinction and non-extinction groups on day 8. The levels of GlyT-1 mRNA in the extinction group were significantly lower than those in the non-extinction group [$t(14) = 3.73$, $p < 0.05$] (Fig. 4b).

3.4. Experiment 3: Extracellular glycine concentration in the hippocampus

In the analysis of extracellular glycine concentration on FC, two-way repeated measures ANOVA showed a significant main effect of time [$F(7, 70) = 8.81$, $p < 0.05$], and interaction between time and stress [$F(7, 70) = 7.26$, $p < 0.05$]. There was no main effect of stress [$F(1, 70) = 0.85$, $p = 0.37$]. Post hoc test revealed a significant difference in the concentration of extracellular glycine between the SPS and sham groups at the time of footshock stimulation [$t(10) = 3.26$, $p < 0.05$]. With respect to FC, there was no significant difference in the extracellular glycine concentration between the SPS and sham groups, except for at the time of footshock stimulation (Fig. 5).

Analysis of extracellular glycine concentration at the time of the first CE showed

that the extracellular glycine concentration in the SPS group was significantly decreased relative to the sham group (Fig. 6). Two-way repeated measures ANOVA showed a significant main effect of stress [$F(1, 70) = 109.48$, $p < 0.05$]. There was no main effect of time [$F(7, 70) = 0.98$, $p = 0.45$], and no significant interaction between time and stress [$F(7, 70) = 1.61$, $p = 0.15$].

4. Discussion

The present study generated the following key findings. First, SPS rats exhibited an increased and sustained contextual fear response. Second, a sustained increase in hippocampal Gly-T1 mRNA levels was induced after FC in SPS rats relative to sham rats, and this was accompanied by a decrease in the extracellular glycine concentration in the hippocampus. Third, extinction training for 7 days significantly decreased the levels of GlyT-1 mRNA in SPS rats relative to rats that did not undergo extinction training, and a significant difference in fear responses was evident between these 2 groups.

We first replicated our recent series of studies indicating that contextual freezing is significantly enhanced in SPS rats during the first context exposure (Imanaka et al., 2006; Iwamoto et al., 2007; Takahashi et al., 2006). Furthermore, the sustained increase

in fear response in SPS rats was consistent with our previous study using a different FC paradigm (Yamamoto et al., 2008).

In SPS rats, we found that the sustained increase in hippocampal GlyT-1 mRNA expression was induced in response to FC. In addition, administration of repeated extinction training markedly reduced the enhancement of GlyT-1 mRNA levels in SPS rats in response to FC. These findings strongly suggest that hippocampal GlyT-1 mRNA was closely associated with the intensity of contextual fear. In addition, the time course changes in both GlyT-1 mRNA and extracellular glycine concentrations in the present study imply that the increased expression of GlyT-1 mRNA play a role in the decreased concentration of extracellular glycine, although the GlyT-1 protein levels were not measured. To our knowledge, this is the first study to demonstrate that the FC paradigm in an animal model of PTSD induces a decrease in the concentration of glycine in the hippocampus. We were not able to determine the precise time at which the initial decrease in extracellular glycine concentrations occurred in the SPS rats. However, our results suggest the following: (1) Administration of footshock in contextual fear conditioning increases the synaptic concentration of glycine in the hippocampus of SPS rats. (2) The transiently abundant release of glycine from the pre-synapse subsequently induces Gly-T1 mRNA expression. (3) The up-regulation of GlyT-1 mRNA reduces the

glycine concentration in the extracellular space.

SPS rats showed higher levels of conditioned fear compared to sham rats 1 day after FC. With respect to the difference in fear acquisition between these 2 groups, it is conceivable that this is due to the difference in the potentiation of NMDAR-mediated signaling through the increase in glycine release in the SPS group, but not in the sham group, at the time of footshock stimulation. Further research is needed to address this issue.

In addition to the impaired fear extinction in SPS rats, we previously reported that DCS ameliorated the impaired fear extinction in SPS rats (Yamamoto et al., 2008). Although several studies in animals and humans have so far shown that DCS enhances fear extinction when given in conjunction with extinction training (Davis et al., 2006; Ledgerwood et al., 2004; Ressler et al., 2004; Walker et al., 2002), the precise underlying mechanism remains to be determined. However, the activation of the strychinine-insensitive glycine-binding site of the NMDAR by DCS, which is a partial NMDAR agonist (Hood et al., 1989; Watson et al., 1990), facilitated NMDAR-mediated synaptic potentials and subsequently facilitated learning and memory (Rouaud and Billard, 2003). Furthermore, DCS has been reported to enhance behaviors related to spatial memory that are closely dependent on the hippocampus (Lelong et al., 2001;

Thompson et al., 1992). In line with its suggested action as a cognitive enhancer, administration of DCS was shown to rescue age-related deficits of cellular mechanisms of learning and memory (Billard and Rouaud, 2007). More recently, there are reports showing that DCS reversed the impairment of learning caused by exposure to stress (Akirav et al., 2009; Waddell et al., 2010). For example, Akirav and colleagues have reported that DCS injected into the basolateral amygdala reversed the impairing effects of stress exposure on the extinction of contextual fear, but did not reverse the effects of conditioned taste aversion (Akirav et al., 2009).

With respect to the extracellular glycine concentration in the hippocampus, it is of interest to consider the mechanism by which DCS with extinction training ameliorated impaired fear extinction in SPS rats in our previous study (Yamamoto et al., 2008). Based on the present result, it is conceivable that hypofunction of hippocampal NMDARs in SPS rats induced by the decreased concentrations of glycine, secondary to the increase in GlyT-1, may be alleviated by DCS, along with amelioration of the impaired fear extinction. Intriguingly, Guastella and colleagues (2007) have shown that DCS may not affect non-clinical fear in human populations. They evaluated the effectiveness of administering DCS as an adjunct to exposure therapy in sub-clinical spider fear subjects; DCS did not enhance the reduction of fear of spiders. In light of

GlyT-1 mRNA expression in the hippocampus, the fact that only SPS, but not sham, rats showed alterations in GlyT-1 mRNA expression after FC may explain why DCS treatment often fails to affect fear extinction in “normal” subjects.

If the impaired fear extinction in PTSD is induced by the glycinergic-mediated hypofunction of NMDARs, in line with the findings in SPS rats, then drugs that stimulate NMDAR may be effective in the treatment of PTSD in conjunction with extinction training. However, competitive NMDAR agonists acting at the glutamate site were reported to have a number of undesirable side effects, such as seizures and neurotoxicity (Danysz and Parsons, 1998). Also, it has been suggested that glycine, a full agonist, may be more effective than the partial agonist DCS (Heresco-Levy and Javitt, 2004). Research is currently focusing on GlyT-1 inhibitors, which increase extracellular levels of glycine, as possible cognitive enhancers. It is of interest that SSR504734, a GlyT-1 inhibitor, has recently been shown to reverse social recognition impairment induced by neonatal phencyclidine treatment in rats (Depoortere et al., 2005; Harich et al., 2007) and enhance pre-pulse inhibition (PPI) in a mouse strain (DBA/2) with intrinsic sensorimotor gating deficiency (Depoortere et al., 2005). So far, the clinical efficacy of SSR504734 has not been reported. Some patients with PTSD exhibit reduced PPI (Grillon et al., 1996), suggesting that co-administration of

SSR504734 with extinction training may be beneficial in the treatment of PTSD. In addition, Singer and colleagues (2009) have demonstrated the promnestic effects of SSR504734 under normal physiological conditions, lending further support to its potential as a therapeutic drug for the treatment of impaired fear extinction (Singer et al., 2009).

Although it is postulated that dysfunctional fear extinction plays an important role in the development of clinical symptoms of PTSD (Milad et al., 2006; Quirk et al., 2006; Rauch et al., 2006; Rothbaum et al., 2003), the precise mechanism of dysfunction remains unknown. The findings of the present study using an animal model of PTSD suggest that the impaired contextual fear extinction observed in PTSD is due to diminished glycine concentrations occurring as a result of increased levels of GlyT-1 mRNA in the hippocampus. If so, administration of DCS with extinction training would be appropriate to ameliorate the impaired fear extinction. In addition, the enhancement of the glycine site on the NMDARs by GlyT-1 inhibitors with cognitive behavioural therapy might also be efficacious for the treatment of PTSD. However, the efficacy of DCS or GlyT-1 inhibitors has yet to be demonstrated; therefore, further studies are needed to promote our understanding of the pathophysiology of PTSD and the development of novel therapeutic strategies for PTSD.

References

Akirav I, Segev A, Motanis H, Maroun M. D-cycloserine into the BLA reverses the impairing effects of exposure to stress on the extinction of contextual fear, but not conditioned taste aversion. Learning & Memory 2009; 16:682-686.

Billard JM, Rouaud E. Deficit of NMDA receptor activation in CA1 hippocampal area of aged rats is rescued by D-cycloserine. The European Journal of Neuroscience 2007;25:2260-2268.

Cukor J, Spitalnick J, Difede J, Rizzo A, Rothbaum BO. Emerging treatments for PTSD. Clinical Psychology Review 2009; 29:715-726.

Danysz W, Parsons CG. Glycine and N-methyl-D-aspartate receptors: physiological significance and possible therapeutic applications. Pharmacological Reviews 1998;50:597-664.

Davis M, Ressler K, Rothbaum BO, Richardson R. Effects of D-cycloserine on extinction: translation from preclinical to clinical work. Biological Psychiatry 2006;60:369-375.

Depoortere R, Dargazanli G, Estenne-Bouhtou G, Coste A, Lanneau C, Desvignes C, et al. Neurochemical, electrophysiological and pharmacological profiles of the selective inhibitor of the glycine transporter-1 SSR504734, a potential new type

of antipsychotic. Neuropsychopharmacology 2005;30:1963-1985.

Grillon C, Morgan CA, Southwick SM, Davis M, Charney DS. Baseline startle amplitude and prepulse inhibition in Vietnam veterans with posttraumatic stress disorder. Psychiatry Research 1996;64:169-178.

Guastella AJ, Dadds MR, Lovibond PF, Mitchell P, Richardson R. A randomized controlled trial of the effect of D-cycloserine on exposure therapy for spider fear. Journal of Psychiatric research 2007;41:466-471.

Guastella AJ, Richardson R, Lovibond PF, Rapee RM, Gaston JE, Mitchell P, et al. A randomized controlled trial of D-cycloserine enhancement of exposure therapy for social anxiety disorder. Biological Psychiatry 2008;63:544-549.

Harich S, Gross G, Bespalov A. Stimulation of the metabotropic glutamate 2/3 receptor attenuates social novelty discrimination deficits induced by neonatal phencyclidine treatment. Psychopharmacology (Berl) 2007;192:511-519.

Heresco-Levy U, Javitt DC. Comparative effects of glycine and D-cycloserine on persistent negative symptoms in schizophrenia: a retrospective analysis. Schizophrenia Research 2004;66:89-96.

Hofmann SG, Meuret AE, Smits JA, Simon NM, Pollack MH, Eisenmenger K, et al. Augmentation of exposure therapy with D-cycloserine for social anxiety

disorder. Archives of General Psychiatry 2006;63:298-304.

Hood WF, Compton RP, Monahan JB. D-cycloserine: a ligand for the N-methyl-D-aspartate coupled glycine receptor has partial agonist characteristics. Neuroscience Letters 1989;98:91-95.

Imanaka A, Morinobu S, Toki S, Yamawaki S. Importance of early environment in the development of post-traumatic stress disorder-like behaviors. Behavioural Brain Research 2006;173:129-137.

Iwamoto Y, Morinobu S, Takahashi T, Yamawaki S. Single prolonged stress increases contextual freezing and the expression of glycine transporter 1 and vesicle-associated membrane protein 2 mRNA in the hippocampus of rats. Progress in Neuro-psychopharmacology & Biological Psychiatry 2007;31:642-651.

Kushner MG, Kim SW, Donahue C, Thuras P, Adson D, Kotlyar M, et al. D-cycloserine augmented exposure therapy for obsessive-compulsive disorder. Biological Psychiatry 2007;62:835-838.

Ledgerwood L, Richardson R, Cranney J. D-cycloserine and the facilitation of extinction of conditioned fear: consequences for reinstatement. Behavioral Neuroscience 2004;118:505-513.

Ledgerwood L, Richardson R, Cranney J. D-cycloserine facilitates extinction of learned fear: effects on reacquisition and generalized extinction. Biological Psychiatry 2005;57:841-847.

Lelong V, Dauphin F, Boulouard M. RS 67333 and D-cycloserine accelerate learning acquisition in the rat. Neuropharmacology 2001;41:517-522.

Liberzon I, Krstov M, Young EA. Stress-restress: effects on ACTH and fast feedback. Psychoneuroendocrinology 1997;22:443-453.

Liberzon I, Lopez JF, Flagel SB, Vazquez DM, Young EA. Differential regulation of hippocampal glucocorticoid receptors mRNA and fast feedback: relevance to post-traumatic stress disorder. Journal of Neuroendocrinology 1999;11:11-17.

Mendes DD, Mello MF, Ventura P, Passarela Cde M, Mari Jde J. A systematic review on the effectiveness of cognitive behavioral therapy for posttraumatic stress disorder. International Journal of Psychiatry Medicine 2008;38:241-259.

Milad MR, Rauch SL, Pitman RK, Quirk GJ. Fear extinction in rats: implications for human brain imaging and anxiety disorders. Biological Psychology 2006;73:61-71.

Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. New York: Academic Press,1986.

Quirk GJ, Garcia R, Gonzalez-Lima F. Prefrontal mechanisms in extinction of conditioned fear. Biological Psychiatry 2006;60:337-343.

Rauch SL, Shin LM, Phelps EA. Neurocircuitry models of posttraumatic stress disorder and extinction: human neuroimaging research--past, present, and future. Biological Psychiatry 2006;60:376-382.

Ressler KJ, Rothbaum BO, Tannenbaum L, Anderson P, Graap K, Zimand E, et al. Cognitive enhancers as adjuncts to psychotherapy: use of D-cycloserine in phobic individuals to facilitate extinction of fear. Archives of General Psychiatry 2004;61:1136-1144.

Rothbaum BO, Davis M. Applying learning principles to the treatment of post-trauma reactions. Annals of the New York Academy of Sciences 2003;1008:112-121.

Rouaud E, Billard JM. D-cycloserine facilitates synaptic plasticity but impairs glutamatergic neurotransmission in rat hippocampal slices. British Journal of Pharmacology 2003;140:1051-1056.

Singer P, Feldon J, Yee BK. The glycine transporter 1 inhibitor SSR504734 enhances working memory performance in a continuous delayed alternation task in C57BL/6 mice. Psychopharmacology (Berl) 2009;202:371-384.

Sur C, Kinney GG. Glycine transporter 1 inhibitors and modulation of NMDA

receptor-mediated excitatory neurotransmission. Current Drug Targets 2007;8:643-649.

Takahashi T, Morinobu S, Iwamoto Y, Yamawaki S. Effect of paroxetine on enhanced contextual fear induced by single prolonged stress in rats. Psychopharmacology (Berl) 2006;189:165-173.

Thompson LT, Moskal JR, Disterhoft JF. Hippocampus-dependent learning facilitated by a monoclonal antibody or D-cycloserine. Nature 1992;359:638-641.

Waddell J, Mallimo E, Shors T. D-cycloserine reverses the detrimental effects of stress on learning in females and enhances retention in males. Neurobiology of Learning and Memory 2010;93:31-36.

Walker DL, Ressler KJ, Lu KT, Davis M. Facilitation of conditioned fear extinction by systemic administration or intra-amygdala infusions of D-cycloserine as assessed with fear-potentiated startle in rats. Journal of Neuroscience 2002;22:2343-2351.

Watson GB, Bolanowski MA, Baganoff MP, Deppeler CL, Lanthorn TH. D-cycloserine acts as a partial agonist at the glycine modulatory site of the NMDA receptor expressed in Xenopus oocytes. Brain Research 1990;510:158-160.

Yamamoto S, Morinobu S, Fuchikami M, Kurata A, Kozuru T, Yamawaki S. Effects of

single prolonged stress and D-cycloserine on contextual fear extinction and hippocampal NMDA receptor expression in a rat model of PTSD. Neuropsychopharmacology 2008;33:2108-2116.

Figure legends

Fig. 1 Treatment groups and procedure. In the sham group, context exposure was performed once daily for 2 days following fear conditioning and the measurement of freeing was conducted during context exposure on days 2 and 3. In the SPS group, 2 different types of experiments were undertaken. In the first study, similarly to the sham group, context exposure daily for 2 days following fear conditioning and the measurement of freeing during context exposure was performed. In another study, context exposure was continued for up to day 8 and the measurement of freeing was conducted during context exposure on day 8, to clarify the relation between contextual fear and the GlyT-1 mRNA expression. In this experiment, SPS rats without extinction training were used as controls. The gray arrows indicate time-points of hippocampal tissue sampling.

Fig. 2 Influence of SPS on contextual fear response. Data are expressed as mean ± SEM of 8 rats per group. SPS rats showed increased freezing responses compared to Sham rats on days 2 and 3. Asterisk denotes significance at the 0.05 level.

Fig. 3 GlyT-1 mRNA levels at several time-points. Data are expressed as the ratio of the

concentration of GlyT-1 to that of GAPDH (GlyT-1/GAPDH) and represent the mean ± SEM of 6 rats per group. Asterisk denotes significance at the 0.05 level.

Fig. 4 Influence of extinction training on contextual fear (a) and the GlyT-1 mRNA expression (b) in the SPS group on day 8. Data are expressed as mean ± SEM of 8 rats per group. The level of GlyT-1 mRNA in the extinction group was significantly decreased compared with that in the non-extinction group and it correlated with the freezing response. Asterisk denotes significance at the 0.05 level.

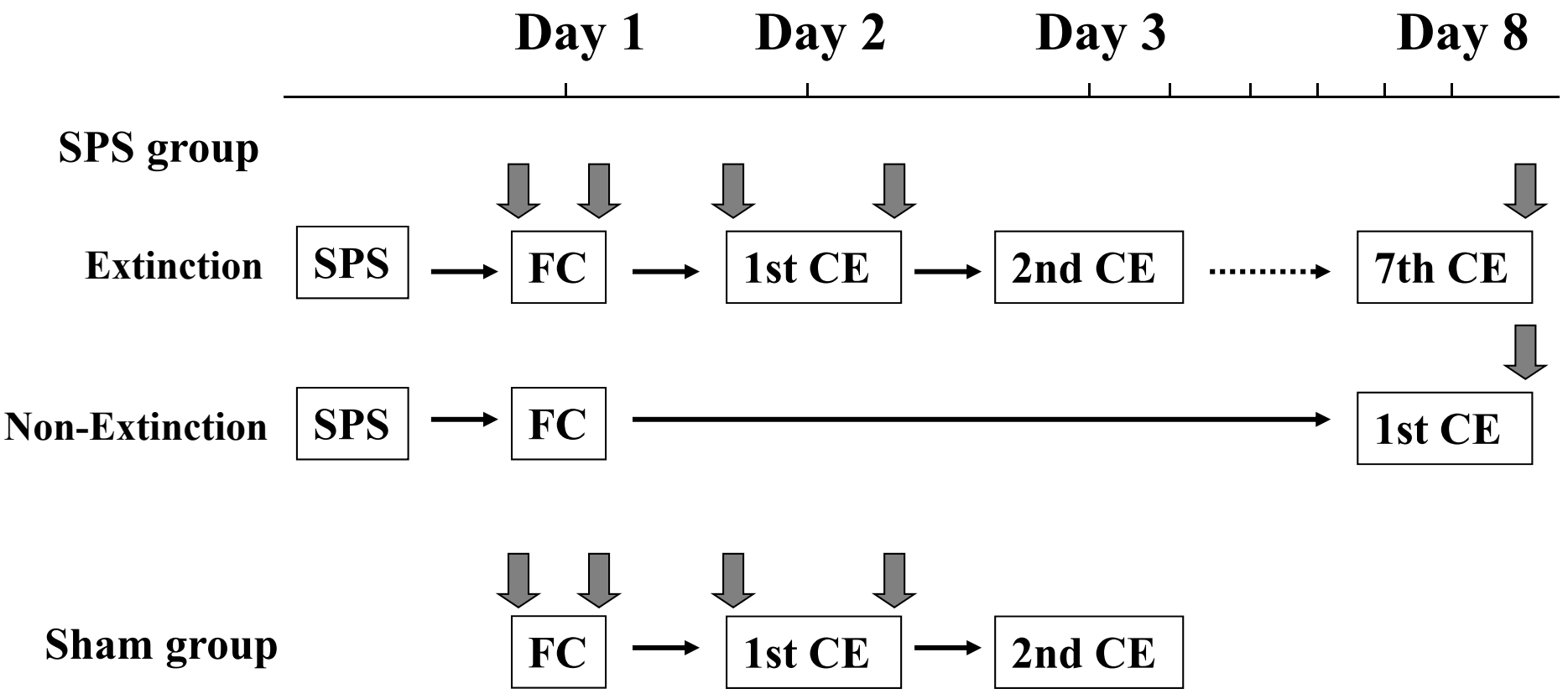
Fig. 5 Concentration of the extracellular glycine in the hippocampus at the time of fear conditioning. Data are expressed as mean ± SEM of 6 rats per groups. There was no significant difference in the extracellular glycine concentration between the SPS and sham groups, except for at the time-point of footshock stimulation. Asterisk denotes significance at the 0.05 level.

Fig. 6 Concentration of extracellular glycine in the hippocampus at the time of the first context exposure. Data are expressed as mean ± SEM of 6 rats per group. The extracellular glycine concentration in the hippocampus in the SPS group was

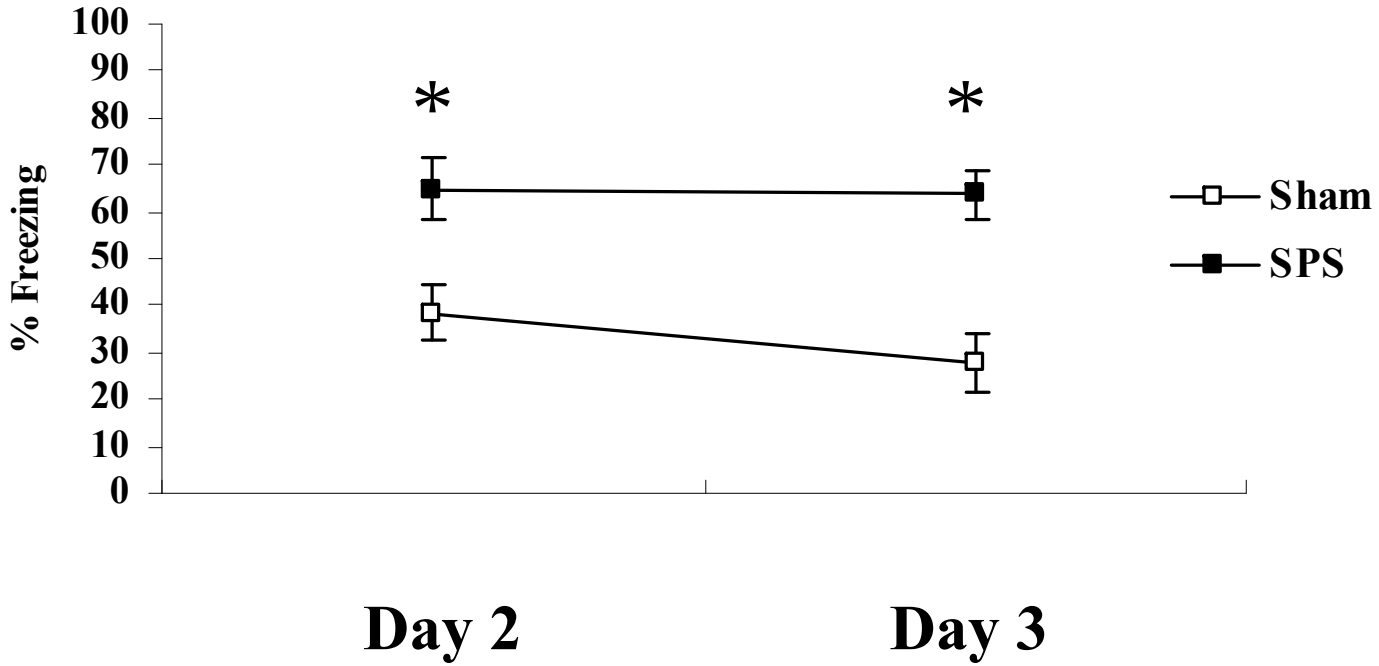
significantly decreased relative to that in the sham group. Asterisk denotes significance at the 0.05 level.

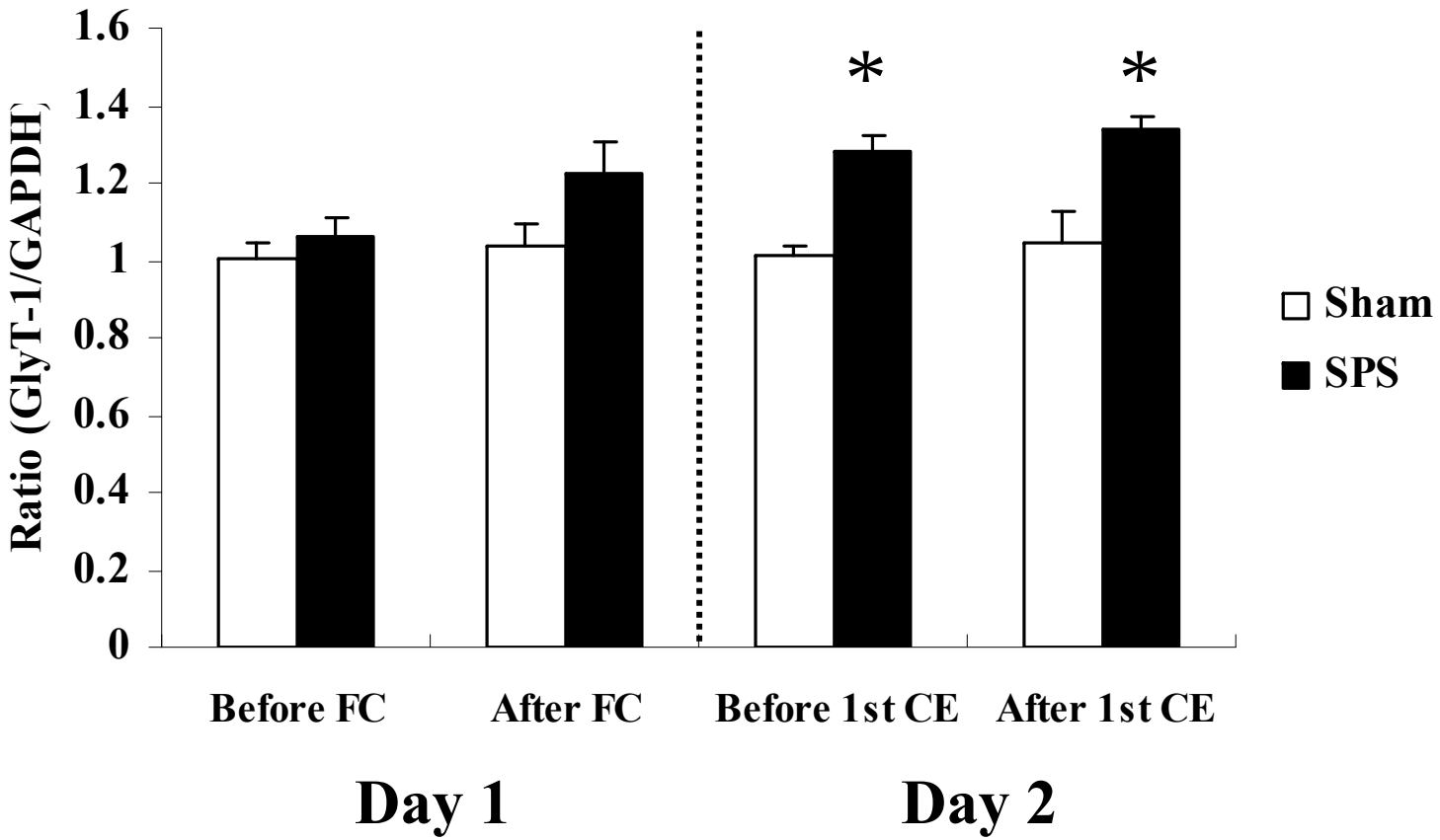
Table 1. Primer and probe sequences used in real-time PCR

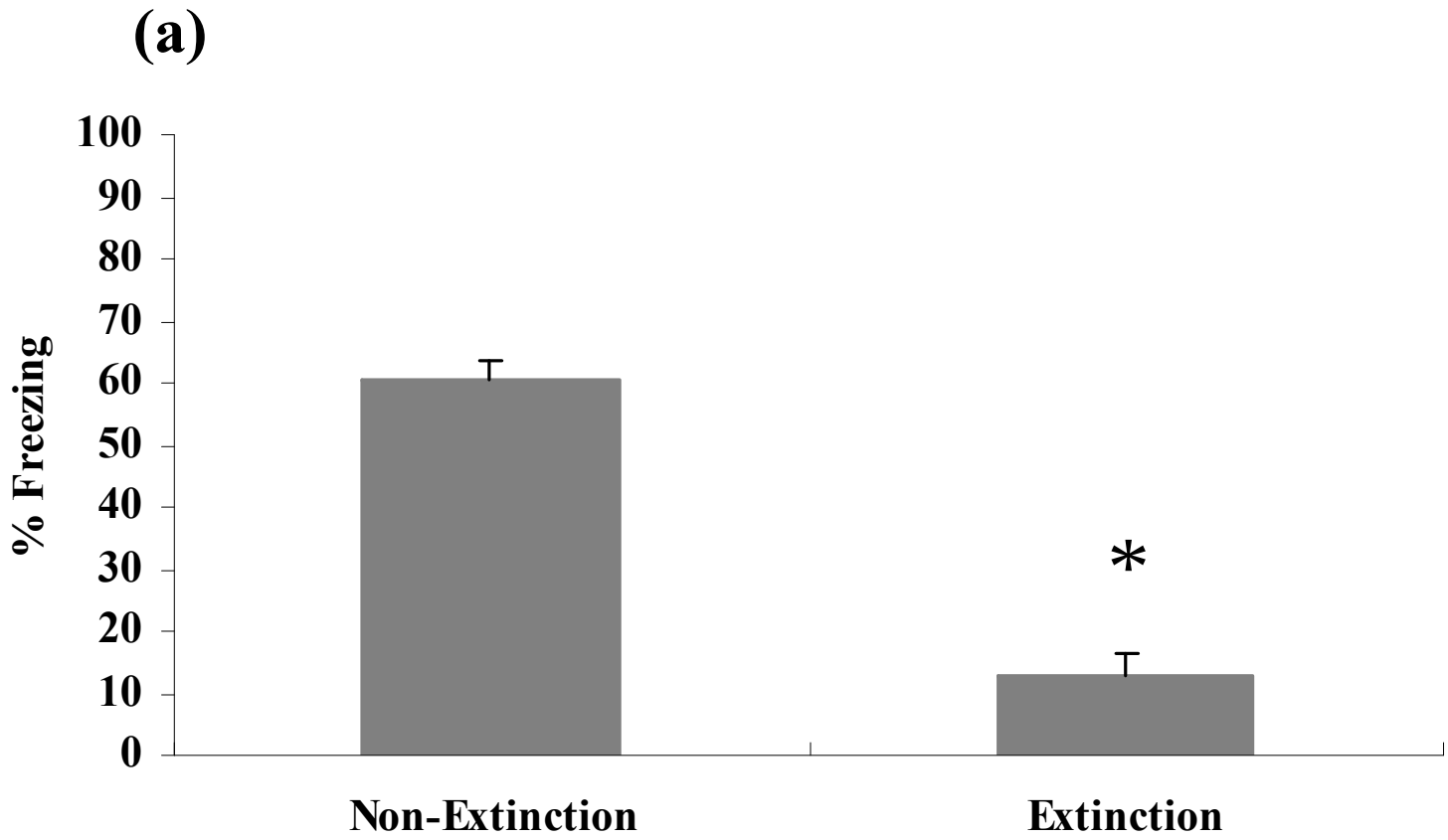
GlyT-1	
Forward primer	**5’-TCCTGAAGATGGGTTTGAGGTT-3’**
Reverse primer	**5’-AGCCGTTACTGCCCACGAT-3’**
TaqMan probe	**5’-FAM-TGCACCCGGACAAGGCCCA-TAMRA-3’**

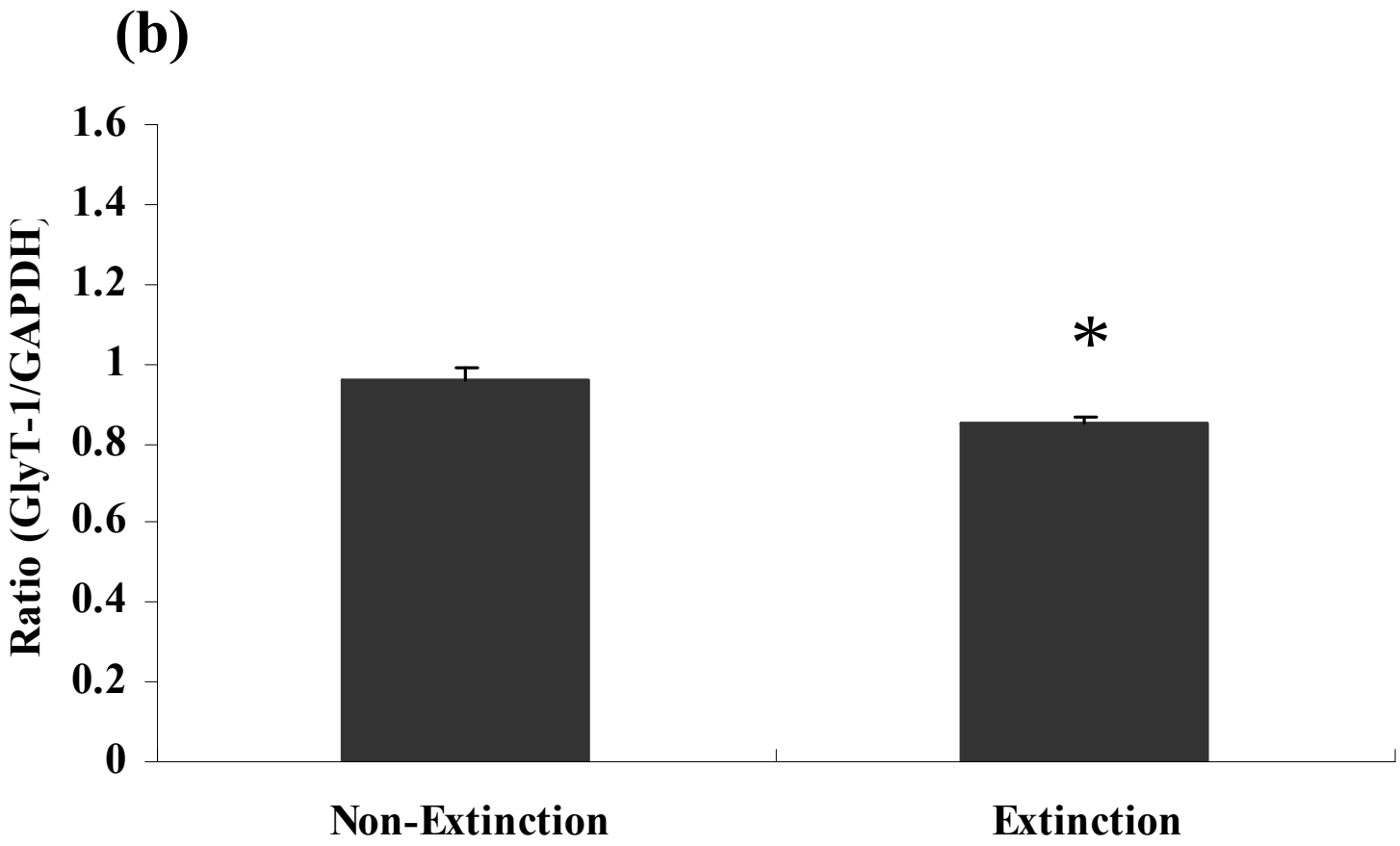


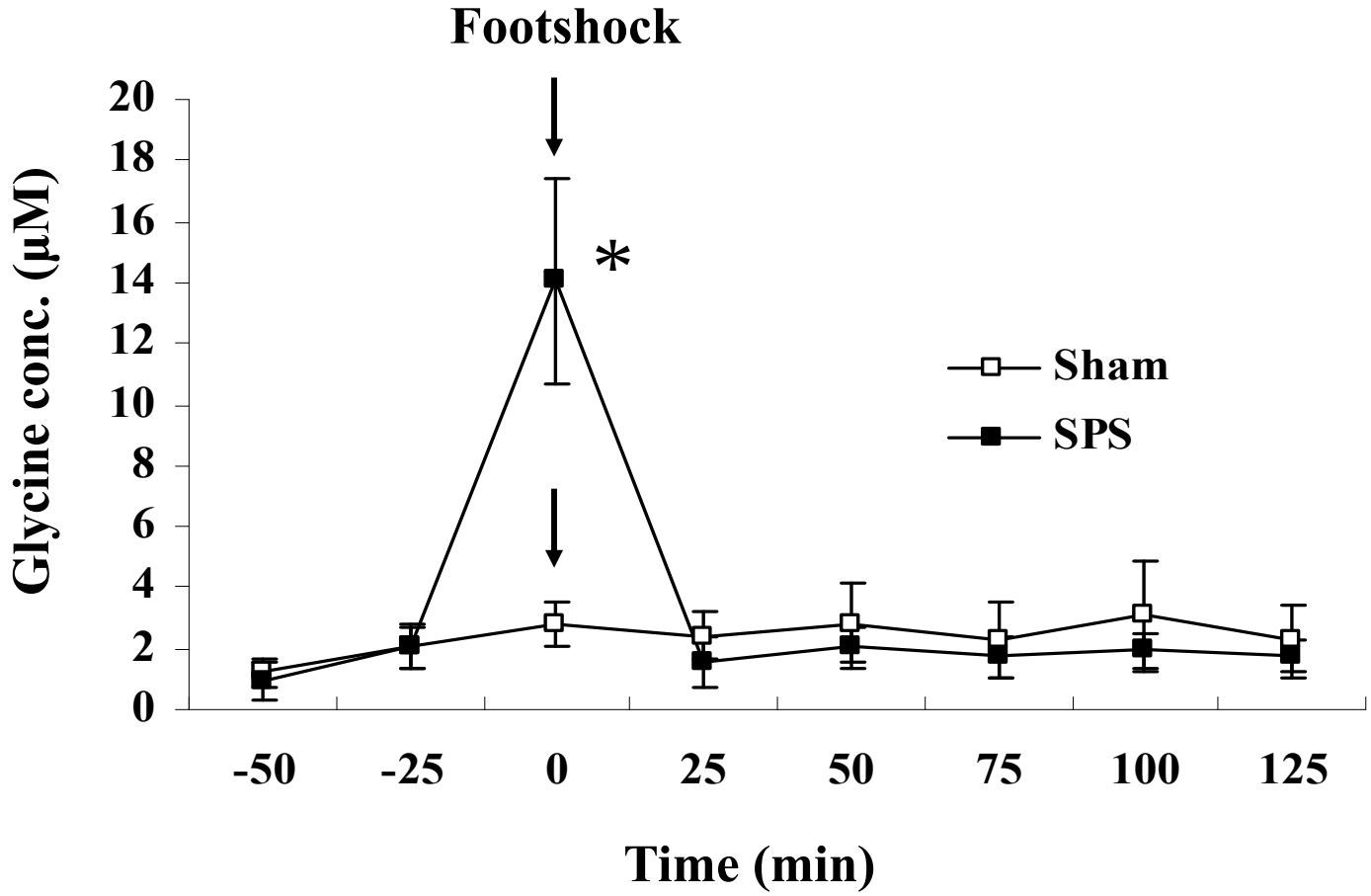
FC: contextual fear conditioning, CE: context exposure

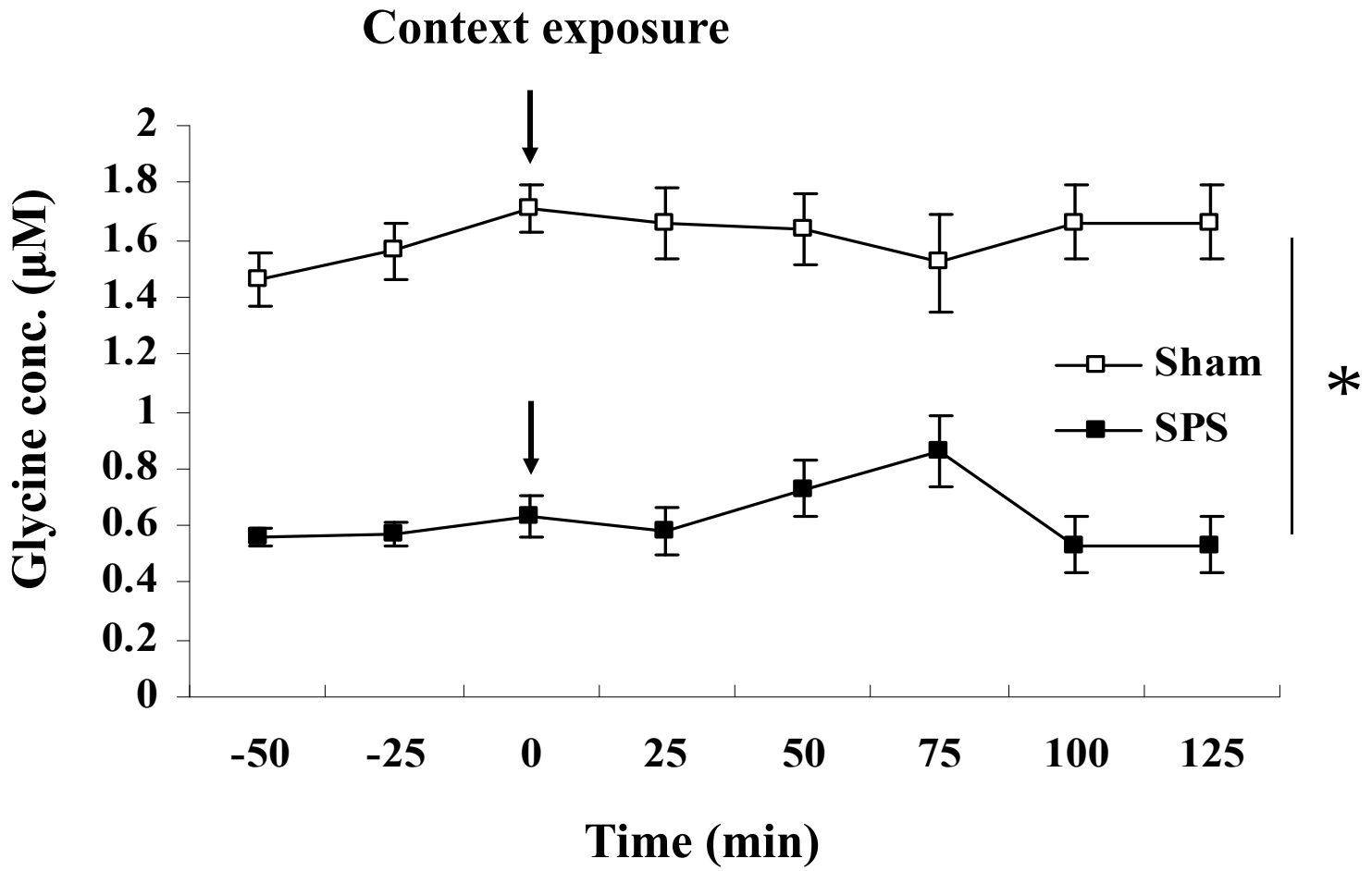

100
90
80
70
60
50
40
30
20
10
0
% Freezing
*
*
Sham
SPS
Day 2
Day 3


1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ratio (GlyT-1/GAPDH)
*
*
Sham
SPS
Before FC
After FC
Before 1st CE
After 1st CE
Day 1
Day 2


(a)
100
90
80
70
60
50
40
30
20
10
0
% Freezing
*
Non-Extinction
Extinction


(b)
Ratio (GlyT-1/GAPDH)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Non-Extinction
Extinction


Footshock
Glycine conc. (µM)
20
18
16
14
12
10
8
6
4
2
0
*
Sham
SPS
-50
-25
0
25
50
75
100
125
Time (min)


Context exposure
Glycine conc. (μM)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-50
-25
0
25
50
75
100
125
Time (min)
Sham
SPS
*