Journal of Psychiatric Research 44 (2010) 1069–1074

Contents lists available at ScienceDirect

Journal of Psychiatric Research

journal homepage: www.elsevier.com/locate/psychires

# 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

ARTICLE INFO

*Article history:*
Received 19 November 2009
Received in revised form
22 March 2010
Accepted 22 March 2010

*Keywords:*
Single prolonged stress
Fear extinction
Glycine
Glycine transporter 1
Hippocampus
Posttraumatic stress disorder

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.


## 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

* Corresponding author. Tel.: +81 82 257 5205; fax: +81 82 257 5209.
*E-mail address:* smoriob@hiroshima-u.ac.jp (S. Morinobu).

0022-3956/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jpsychires.2010.03.013

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. (1997, 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-s 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 s 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.

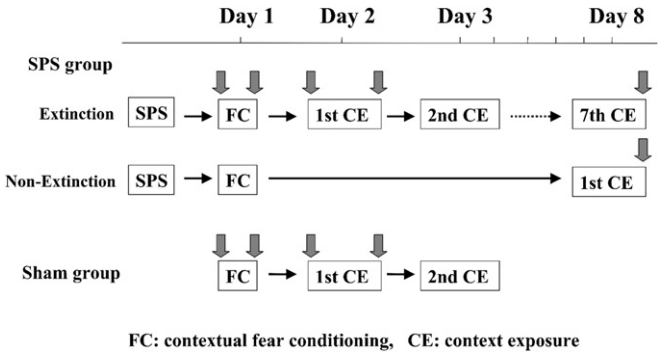


**Fig. 1.** Treatment groups and procedure. In the sham group, context exposure was performed once daily for 2 days following fear conditioning and the measurement of freeing was conducted during context exposure on days 2 and 3. In the SPS group, 2 different types of experiments were undertaken. In 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.