Impact of Post-training REM Sleep Deprivation on Retention of Pavlovian Trace and Contextual Fear Memory in Rats

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4637409038 NARUN PORNPATTANANANGKUL: IMPACT OF POST-TRANING REM SLEEP DEPRIVATION ON RETENTION OF PAVLOVIAN TRACE AND CONTEXTUAL FEAR MEMORY IN RATS, Advisors: Assoc. Prof. NAIPHINICH KOTCHABHAKDI, Assist. Prof. PANRAPEE SUTTIWAN, Co-Advisor: Assoc. Prof. SOMPOCH IAMSUPASIT, 32 pp.

This study aimed to investigate the effects of post-training REM sleep deprivation (RSD) on memory consolidation for hippocampus-dependent fear memory, as indexed by retention. To determine whether the strength of learning interferes with the RSD effects, the RSD rats and the control ones were trained in either a single-trial or three-trial trace fear conditioning (a weak vs. a strong learning task, respectively). Immediately after that, only the RSD rats, not the control ones, were sleep deprived by the flowerpot technique. In the following day, to test whether effects of RSD on memory are modality-specific, both groups of rats were tested for two types of hippocampus-dependent memory: contextual and trace fear memory (for spatial vs. auditory modalities, respectively). Both trace and contextual fear memory of the RSD rats were found to be significantly weaker than those of the control rats only in the three-trial condition, but not in the single-trial condition. The results indicated that: (1) post-training RSD impaired retention of hippocampus-dependent memory, (2) this effect was not modality-specific, and (3) the effect of post-training RSD seemed to be moderated by the strength of learning. By cooperating Silvestri's (2005) results, it appears that post-training RSD is perceivable only with a learning task at a moderate strength, but not with too weak, nor too strong strength. Several neural substances, including protein kinases, ACh and 5-HT, were discussed as possible mechanism underlined this retrograde amnesia effect of RSD in this study.

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Student's signature.....

Advisor's signature.....

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Co-Advisor's Signature.....

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Effects of sleep deprivation (SD) on learning and memory have long been investigated in various contexts in both human and animal studies (for review, see Walker, & Stickgold, 2006; Peigneux, Laureys, Delbeuck, & Maquet, 2001). Even though SD seems to have a strong relationship with learning and memory, its influences have been found to be varied depending on several factors. Effects of some factors are quite robust across studies. For example, a number of studies agree that there are specific time windows for SD to have an effect on learning and memory; SD in other periods in time does not have much effect (Smith & Rose 1996, 1997; Graves, Heller, Pack, & Abel, 2003; Fu et al., 2007). Effects of other factors, however, remain unclear. These include which particular sleep stages are deprived and what a specific learning task is used.

The stages of sleep can be divided to a rapid eye movement (REM) or paradoxical stage and a non-rapid eye movement stage (NREM; Hobson & Pace-Schott, 2002). Compared to NREM, during REM brain waves and eye movements are more active, but muscles seem to be completely inactive. Additionally, there are some neurochemical changes in cholinergic and serotonergic systems occurring in the hippocampus during REM, including an increase in acetylcholine (ACh) and a decrease in serotonin (5-hydroxytryptamine; 5-HT), respectively (for review, see Graves, Pack & Abel, 2001). Some manipulation techniques, called prolonged or total sleep deprivation (TSD), were designed to deprive both stages, e.g., "the gentle stroking" (Ledoux, Sastre, Buda, Luppi, & Jouvet, 1996) and "keeping presenting novel objects" (Guan, Peng & Fang, 2004). Others techniques, called REM sleep deprivation (RSD), were designed to selectively deprive only the REM stage, but not the NREM stage, e.g., "the flowerpot technique." In this particular technique, an animal, during its sleep period, is placed on an inverted flowerpot that is small relative to its body size and surrounded by water. As its muscle becomes atonic during REM, the animal falls down from the flowerpot, get wet and,

hence, be waken up (Youngblood, Zhou, Smagin, Ryan & Harris, 1997). An effectiveness of this technique has been demonstrated in studies that used electroencephalographic (EEG) and electromyographic (EMG) measures to indicate each sleep state (Smith & Gisquet-Verrier, 1996; McDermott et al., 2003). The RSD group showed significantly less REM sleep, but not NREM sleep, than the control group.

It is generally thought that, whereas pre-training SD interferes with memory encoding, post-training SD interferes with memory consolidation (Walker & Stickgold, 2006). Memory consolidation is a process that the short-term memory encoded previously from a learning task transforms to the long-term memory. Investigators have consistently found the strong relationship between memory consolidation and REM (for review, see Smith, 1996; Walker & Stickgold, 2004; Sutherland & McNaughton, 2000). Firstly, during REM, CA1 cells in hippocampus display firing patterns resembling in temporally structured to the firing patterns in previously learned tasks during awake (Louise & Wilson, 2001; Skaggs & McNaughton, 1996). Secondly, it is well documented in rodent studies that REM would increase following an acquisition of different learning tasks, including a hidden-platform version of the Morris water maze (Smith & Rose, 1997), a two-way shock-avoidance task (Smith, Young & Young, 1980) and a complex Y-maze shock- avoidance task (Smith, Kitahama, Valatx, & Jouvet, 1974). Moreover, Smith et al. (1980) also pointed out that the greater amount of REM could predict the better learning animals would be at that task. Finally, enriched environment led rats to increase duration of REM sleep and improves a recall of a brightness discrimination task 28 days after acquisition (Gutwein & Fishbein, 1980a, 1980b). However, REM seemed to be reduced in Alzheimer patients, whose learning and memory were usually attenuated (Montplaisir, Petit, Gauthier, Gaudreau & Décary, 1998).

One way to examine consolidation of memory is by looking at retention tests of that memory. When examining SD effects, many studies have employed learning tasks mediated by the hippocampus since this structure has been found to involve in consolidation and encoding

of many types of memory (Sweatt, 2003). Spatial learning and Pavlovian fear learning are among types of learning that have often been utilized to investigate hippocampal involvement (Whishaw, 1985; LeDoux, 1995, 2000). To investigate spatial learning, the Morris water maze can be used, in which an animal is required to locate a platform over water after several trainings. Employing this paradigm in rats, Smith and Rose (1996) demonstrated that post-training RSD impaired retention of memory only in the hidden-platform version, a hippocampus-dependent task, but not in the visible-platform version, a hippocampal-independent task. Moreover, similar results were found with pre-training TSD (Guan et al., 2004). Nevertheless, this technique has a least one potential flaw when using to study effects of SD. Besides learning ability, motor performance, which might also be attenuated by SD, is required in the task (Ruskin, Liu, Dunn, Bazan, & LaHoste, 2004). Fortunately, this is not the case for Pavlovian fear conditioning.

Another advantage of Pavlovian fear conditioning when applying to study SD effects is that it is relatively quick to learn (Fanselow & Poulos, 2005). Indeed, it has been found that organisms can acquire fear conditioning from a single-trial conditioning (Fanselow & Bolles, 1979). Hence, researchers can do some manipulations, including SD, after one session of conditioning and test this learning in a following session, as a retention test, by looking at conditional fear responses including freezing behavior (Blanchard & Blanchard, 1969; Fanselow, 1980). Several paradigms of Pavlovian fear conditioning can be employed. In the single cued conditioning (aka, delay conditioning) paradigm, animals are paired a single cue (CS; conditional stimulus), e.g., a tone or light, with an aversive stimuli (US; unconditional stimulus), e.g., footshock. In contrast, there is no discrete stimulus presented in the contextual conditioning paradigm; a context itself acts as a CS to predict an US. Lesion studies have showed that, although both require the amygdala, contextual conditioning, but not single cued conditioning, requires the hippocampus (Phillips & LeDoux, 1992; Maren, Aharonov &

Fanselow, 1997) The neural circuit for Pavlovian fear conditioning underlining different fear conditioning paradigms displays in Figure 1.

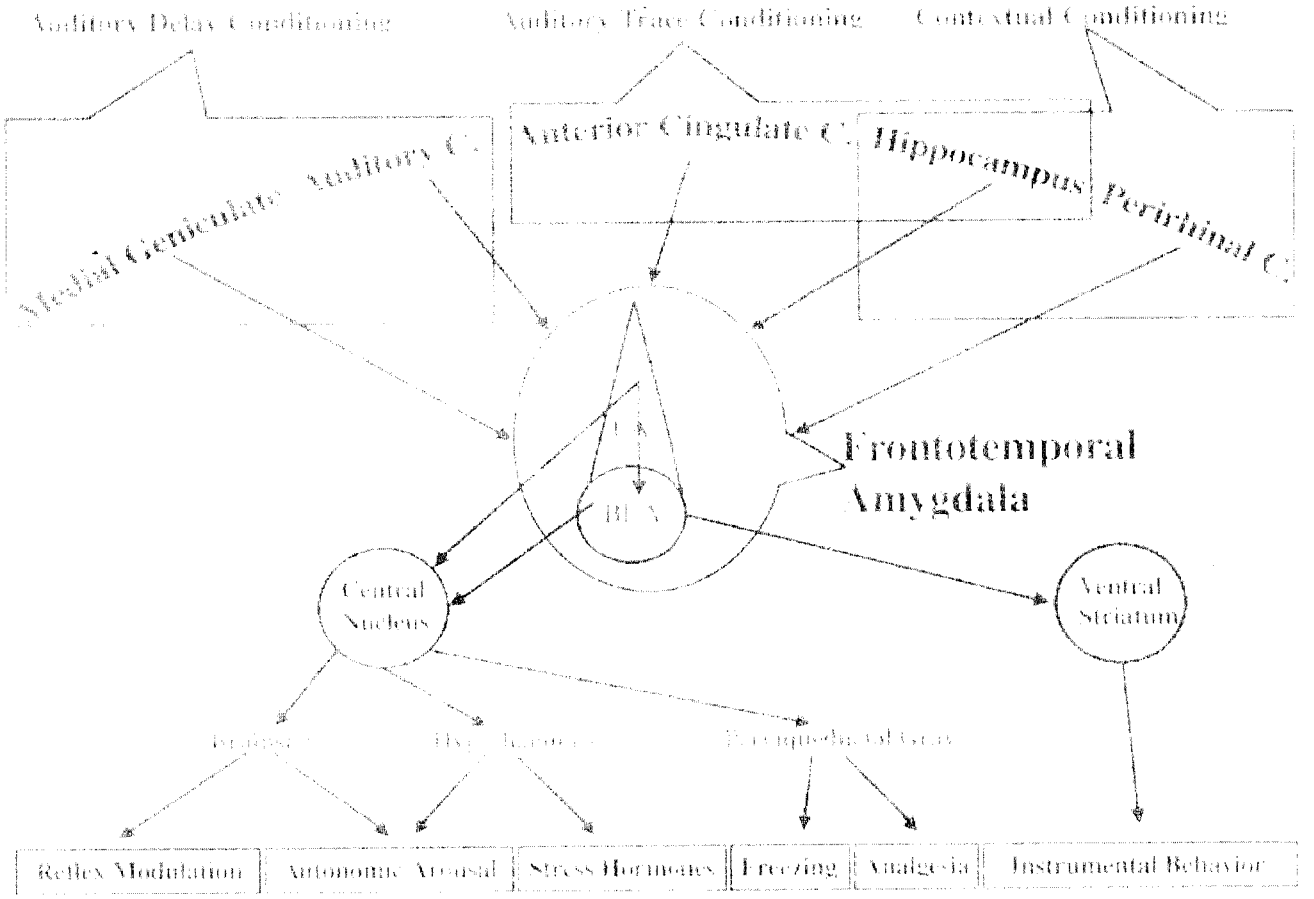

Figure 1. The neural circuit for Pavlovian fear conditioning (adapted from Fanselow & Poulos, 2005) organized according to different fear conditioning paradigms at the top and conditional fear responses at the bottom. LA, the lateral nucleus of the amygdala; BLA, the basolateral nucleus of the amygdala. The BLA and the LA comprise the frontotemporal amygdala.

Trace conditioning is another Pavlovian fear conditioning paradigm that can be used to investigate SD effects. Similar to single cued conditioning, animals are paired a CS with an US in this paradigm; however, there is a time interval, "trace," between CS offset and US onset in which no stimuli is introduced. If the animals have a memory of the task, they would show conditional fear responses during this trace period. Similar to contextual conditioning, but not to single cued conditioning, lesion studies have demonstrated that trace conditioning is hippocampus-dependent (McEchron, Bouwmeester, Tseng, Weiss & Disterhoft, 1998; Quinn,

Oommen, Morrison & Fanselow, 2002; Yoon & Otto, 2007; Chowdhury, Quinn, Fanselow, 2005). Nonetheless, there are some differences between trace conditioning and contextual conditioning. Although the hippocampus and the amygdala are required in both, anterior cingulated cortex is required only in trace conditioning, but not in contextual conditioning (Han et al., 2003). Additionally, nature of CS in the two conditionings requires different modalities, in that a CS in contextual conditioning requires spatial modality, whereas that in trace conditioning requires auditory modality.

Graves et al. (2003) demonstrated in a mice study that post-training TSD selectively impairs retention of memory for contextual conditioning, but not for single cued conditioning. In their study, after being trained in a single trial of single cued conditioning, mice were subjected to TSD and tested for freezing behavior 24h later. Retention for contextual conditioning was tested in the same context without the cue presented, and for single cued conditioning in an altered context, but with the cue presented. Indeed, this finding was in line with studies utilizing pre-training TSD (Ruskin et al., 2004) and pre-training RSD (McDermott et al., 2003) in which SD was also found to selectively impair learning for contextual conditioning. Results from these studies, along with studies that investigated spatial learning using the Morris swimming maze (Smith & Rose, 1996), led many (e.g., Ruskin et al.; Guan et al., 2004; McDermott et al.) to conclude that SD selectively affects hippocampus-dependent learning and memory. Several biological mechanisms underpinning amnesia effects of SD have been proposed, including alterations in LTP, protein kinases signaling, cholinergic systems and serotonergic systems (McDermott et al.; Graves et al., 2003; Graves et al., 2001).

A recent study of Silvestri (2005), however, did not support the notion that SD selectively affects retention of memory of hippocampus-dependent tasks. In her study, post-training RSD did not impair retention for either single cued conditioning or contextual conditioning. From this result, contrary to TSD effects, RSD seemed to have no effect on retention of fear conditioning of any paradigms. Note that, methodological differences between

works of Silvestri and of Graves et al. (2003) were not only at SD manipulations utilized (i.e., RSD vs. TSD, respectively), but also at trials of pairing between the CS and the US in the acquisition phase (i.e., 10 trials vs. a single trial, respectively). Hence, one alternative explanation of the discrepancy between results of the two studies was that stringent learning in Silvestri's animals (cf. Graves' et al. animals) did not permit SD to have much effect. In fact, this suggested that effects of SD might be moderated by the strength of learning tasks. Additionally, in Silvestri's study, the animals were allowed to have another day of resting after RSD until the time of the test for retention, memory consolidation of the CS could occur during this time.

So far, studies employed fear learning paradigms have investigated SD effect on hippocampus by comparing SD effects on retention of single cued conditioning with that on contextual conditioning, as hippocampal-independent and hippocampus-dependent learning, respectively (e.g., Graves et al., 2003; Ruskin et al., 2004; McDermott et al., 2003; Silvestri, 2005; Fu et al., 2007). The difference of SD effects found in such comparison, nonetheless, could also reflect the difference in modalities of CS involved in the two conditionings, as well as brain structures underlining them. It is possible that SD might selectively interfere with learning and memory of conditioning paradigms involving spatial modality like contextual conditioning (and also a hidden-platform version of the Morris swimming maze), but not so in those involving other modalities, including auditory, like single cued and trace conditioning.

The main aim of the present study is to investigate the role of post-training RSD on memory consolidation for hippocampus-dependent fear conditioning, as indexed by retention tests, by adapting protocols of Graves et al. (2003) and Silvestri (2005). To see whether the strength of learning interferes with the RSD effects, the rats were trained in either a single-trial (for weaker learning) or three-trial (for stronger learning) trace fear conditioning, using a tone as a CS, and footshock as an US. By comparing fear memory of the rats conditioned in a single trial with that of the rats conditioned in three trials, a manipulation check of the strength of

learning could be done. Immediately after the acquisition, the RSD group was sleep deprived by the flowerpot technique, but the control group was not. Inhibiting REM sleep after acquisition of the hippocampus-dependent task was designed to disturb memory consolidation process of the RSD rats. To reduce the possibility that the animals might consolidate memory in the day after the acquisition phase as in Silvestri's study, a time gap between acquisition and retention-testing phases was 24h, instead of 48h. To test whether effects of RSD on memory are modality-specific, trace memory was examined along with contextual memory in the retention-testing phase. This is because, although trace conditioning is hippocampus-dependent (like contextual conditioning), it requires auditory, instead of spatial, modality. Particularly, in the retention-testing phase, the rats were tested for freezing behavior in two different contexts: (1) in the same context with the acquisition phase without the CS presented for testing contextual memory and (2) in an altered context with the CS presented for testing trace memory.

Overall effects of RSD on trace and contextual fear memory could lead to three possible patterns of results with different rationales. It is firstly hypothesized that, if post-training RSD selectively interferes with retention of hippocampus-dependent fear memory like what Graves et al. (2003) found in TSD, both trace and contextual fear memory should be impaired in the RSD group, but not in the control group, as indicated by less freezing behavior in the retention-testing phrase. It is secondly hypothesized that, if post-training RSD does not interfere with retention of fear memory regardless of hippocampal dependence (or independence) like what Silvestri (2005) found, there should be no difference in contextual and in trace memory between the RSD and the control group, as indicated by similar freezing behavior in the retention-testing phrase. It is thirdly hypothesized that, if post-training RSD selectively interferes with retention of fear memory that involves spatial modality, only contextual memory, but not trace memory, would be impaired in the RSD group, but not in the control group, as indicated by less freezing behavior in the retention-testing phrase. Additionally, as it would be easier to consolidate memory of stronger learning tasks, it is

hypothesized that, overall, rats in the single-trial condition should have weaker trace and contextual fear memory than those in the three-trial condition, as indexed by freezing behavior in the retention-testing phrase. Finally, it is hypothesized that, if the effect of RSD is mediated by the strength of a learning task, there should be an interaction between sleep deprivation (RSD vs. control) and the number of trials conditioned (single trial vs. three trials). That is, RSD should differently impair memory learned from conditioning tasks with different trials.

Method

Subjects

Twenty-four male Sprague-Dawley rats, weighting 250 g upon arrival, were obtained from the national laboratory animal centre, Mahidol University, Nakhon Pathom, Thailand. To become acclimatized to the laboratory, they were housed individually for one week before beginning of behavioral procedures. Hence, they weighted 275-300 by that time. A 12:12 h light/dark cycle with lights on at 6 am (Zeitgeber Time' [ZT] 0) and off at 6 pm (ZT'12) was maintained. Food and water were provided *ad libitum*.

Material

The flowerpot technique was done by placing the rats onto an inverted flowerpot, either having 7.9 cm (for the RSD group) or 21.5 cm (for the control group) diameter, located at the bottom of a large plastic garbage pail (42 cm Base x 46cm Height). The diameter of the flowerpot used in the RSD group was consistent with that of Smith and Gisquet-Verrier (1996). Water was filled up to 1 cm below the level of the platform and changed after each use. To reduce effects from temperature changes, the pail was placed in the same room with the rats' home cages.

For conditioning, two distinct chambers were used (chamber A and B for context A and B, respectively), which were made from two identical rectangular conditioning chambers (28cm Wide x 25cm Long x 33cm Height) with rear walls and front door made of clear Plexiglas, two sidewalls made of aluminum (Coulbourn Instruments, Lehigh Valley, PA, Model Habitest, E10-10). The chamber floor contained 16 stainless steel rods. Each chamber was located in the same darkened sound-attenuating cubicle (64.5cm W x 49.5cm L x 54cm H; Coulbourn Instruments). An infrared video camera monitor system attached to the cubicle was used to observe rat behaviors. A ventilation fan attached to the cubicle provided a background noise of ~50 dB.

To be distinguish from the chamber A, the chamber B was manipulated by (1) covering its floor with green plastic net, (2) covering its front door and two sidewalls with green plastic net placed over red plastic board, (3) turning on a 5W red light bulb, which was located behind one of the sidewalls. Additionally, to alter olfactory cues in the two contexts and to remove stress odor, the chamber A and B were cleaned with 5% acetic acid and 70% ethanol, respectively, before and after each session.

To minimize any effects of head orientation on sound intensity, a piezoelectric speaker (Future Kit, Bangkok, Thailand, Model FB03) was attached on the center top of the cubicle. The frequency and loudness of the acoustic CS (4 kHz, 80 dB) were controlled by a customized modified signal generator (Future Kit, Model FA903) and power amplifier (Future Kit, Model FK602), respectively. They were confirmed by a meter in C scale (Larson Davis Laboratories, Precision integrating sound level meter, Model 800B) placed on the floor of the chamber directly below the speakers.

The US was footshock (0.9 mA) delivered via grid floor by a precision regulated animal shocker (Coulbourn Instruments, Model E13-14). Stimulus presentations were controlled by a custom written electronic circuitry connected with four timers (Campden Instruments LTD): two sets of Predet. Counter 213 for timing pre-CS and CS presentation intervals, Reinforcement Timer 245 for timing trace interval and Process Timer 343 for timing US presentation interval.

Design and Procedures

The experimental design involved two between-subjects factors (sleep deprivation [RSD vs. control] and the number of trials conditioned [single trial vs. three trials]) and two with-in dependent measures (trace memory and contextual memory). The rats were randomly assigned to one of four groups according to the between-subjects factors with six rats in each group. All behavioral procedures covered on two consecutive days. Figure 2 displays the chronological depiction of the experimental protocol.

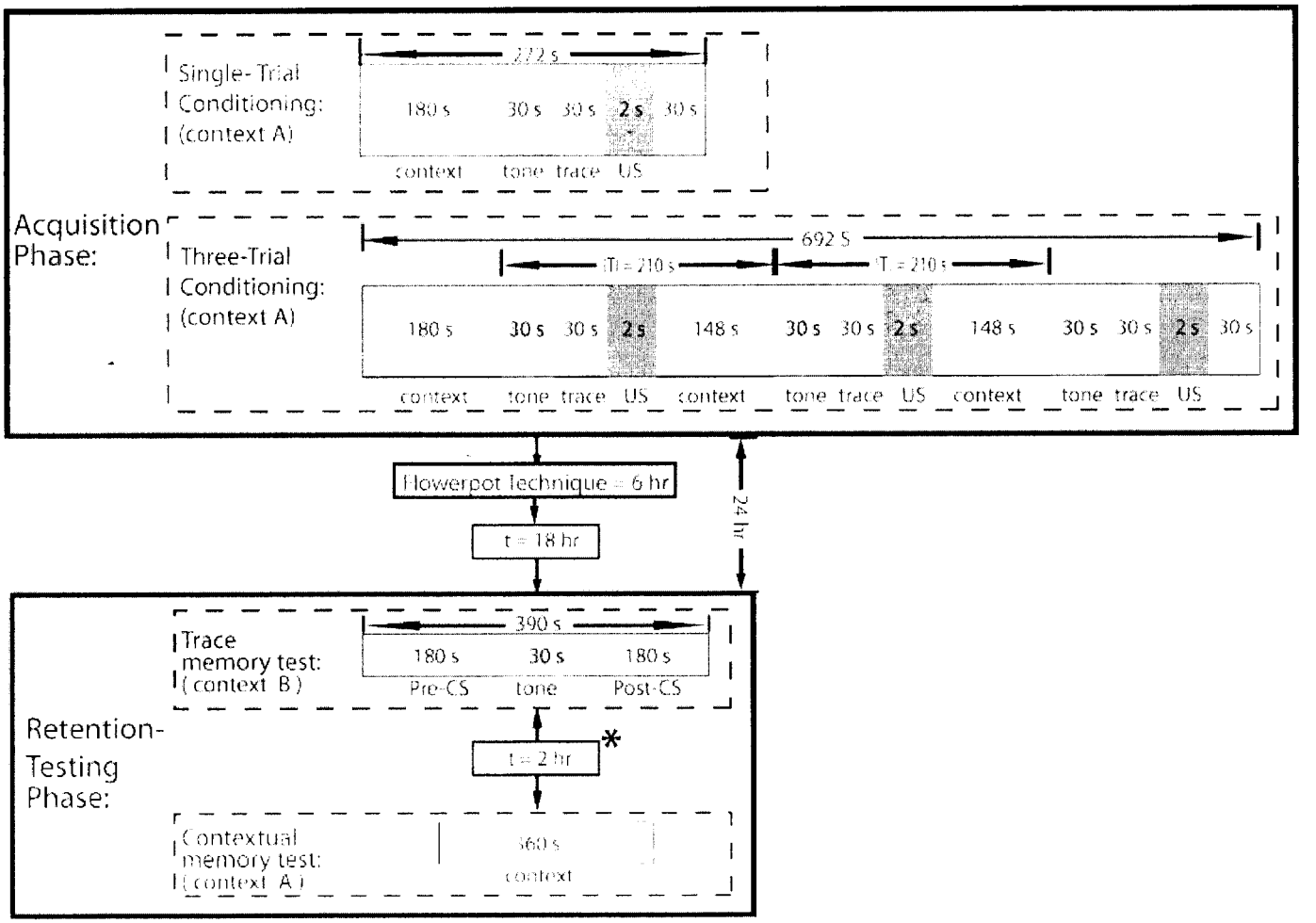

Figure2. Chronological depiction of the experimental protocol. In the single-trial conditioning, after staying for 180 s, the rats were presented with a 30-second tone (CS) followed by a 30-second trace interval, and then, by a 2-second footshock (US). This timing structure was also used in the three-trial conditioning, but there were other two subsequent pairings after the first one with 210-second ITI. Thirty seconds later, the flowerpot technique was manipulated for six hours. Then, the rats rested in their homecase for 18 hours, before the beginning of the retention-testing phase which consisted of two tests. In the trace memory test, a 180-second undisturbed period (Pre-CS) was followed by a 30-second tone (CS), and then, by another 180-second undisturbed period (Post-CS). In the contextual memory test, there was a 360-second undisturbed period (Context). * The order of the two tests was counter-balanced across groups. Parentheses indicate the contexts where the experiment took place. ITI, inter-stimuli interval; Context A, a context where the shock occurred; Context B, an altered context; Trace, a period

between the offset of the tone and the onset of the US; Context, a period where there were no stimuli presented; CS, conditioned stimulus (tone); US, unconditioned stimulus (footshock).

In the acquisition phase, which began at ZT'5 (Graves et al., 2003), the rats were trained in a trace fear conditioning in the context A. In the single-trial conditioning, after being placed into the chamber A for 180 seconds, each rat was presented with a tone (CS) for 30 seconds. Then 30 seconds later (trace interval), there was footshock (US) delivered for 2 seconds. It has been previously found that acquisition of trace fear conditioning with trace interval of 30, but not of 1 – 10, seconds requires hippocampus (Misane ei al., 2005). In the three-trial conditioning, the same timing-structure protocol with a single-trial conditioning was used, but there were other two subsequent pairings after the first one with 210-second inter-stimuli interval (ITI; CS onset to CS onset).

After being left undisturbed in the chamber for another 30 seconds, the rats were taken to another room for the flowerpot technique to take place for six hours. The time and duration of the technique manipulation conformed to specific time window found by Fu et al. (2007). The RSD group was placed in a small platform. To reduce confounding effects of stress to memory processing as a result of the flowerpot technique (Dametto, 2002), the control group was placed in a large platform instead of their home cage. After that, the rats were removed, dried off with towel, and returned to their home cage, where they were allowed to sleep freely.

The retention-testing phase, in which rats' freezing behavior was recorded and measured, began approximately 24 hours after the conditioning (~ZT'5). There were two testing tasks with a two-hour time gap in between. The order of the two tests was counterbalanced across groups. The rats returned to their home case during the gap. Trace memory was measured in a novel context (context B) in which the tone was presented for 30 second. Before the tone, the rats were exposed to this novel context without stimuli presented for 180 seconds (pre-CS period). Rats' behaviors during this pre-CS period reflected baseline behaviors since they were not elicited by any previously paired stimuli. After the tone, the rats

were left undisturbed to test for another 180-second period (post-CS period). Rats' behaviors during this post-CS period indicated trace memory since they were elicited by the previously paired auditory cue. Contextual memory was measured in the same context with the acquisition phase (context A). This test was performed for 360 seconds without CS or US presented (context period). Rats' behaviors during this period revealed contextual memory since they were elicited by the previously paired context.

Defined as an absence of observable movements except for respiration (Fanselow, 1980; Blanchard & Blanchard, 1969), freezing behavior was used as an index of fear memory of the rats and was measured by the time-sampling technique (Fanselow & Bolles, 1979; Bolles & Riley, 1973). The behavior was scored as either freezing or active every 10 seconds for the duration of the pre-CS, the post-CS and the context periods. Freezing and active had a value as one and zero, respectively. The total raw scores from each phrase were transformed into percent freezing by dividing them by the total number of observations taken in each phase (i.e., pre-CS, post-CS, context = 18, 18, 36, respectively), and multiplying that value by 100.

Results

Descriptive statistics for percent freezing at different periods are shown in Table 1. To control for any nonspecific impacts of SD on fear memory, delta scores for trace and contextual fear memory ("delta trace" and "delta context", respectively) were calculated (Paylor, Tracy, Wehner & Rudy, 1994; Graves, 2003, Valentinuzzi et al., 2001). Calculated by subtracting percent freezing in the pre-CS period from that in the post-CS period, a delta trace score indicated CS-specific freezing behavior. Calculated by subtracting percent freezing in the pre-CS period from that in the context period, a delta context score indicated context-specific freezing behavior. Descriptive statistics for these delta scores are shown in Figure 3 for the delta cue and Figure 4 for the delta context.

Table 1

Mean percent freezing (in percent) at different periods by sleep deprivation and by the number of trials conditioned

	Pre-Cue	Post-Cue	Context
Sleep deprivation	*M* (*SD*)	*M* (*SD*)	*M* (*SD*)
		Single Trial	
RSD	0 (0)	18.52 (10.92)	14.82 (8.55)
Control	1.85 (2.87)	32.42 (16.64)	19.44 (7.23)
		Three Trials	
RSD	0.93 (2.27)	21.30 (12.38)	15.28 (7.81)
Control	4.63 (5.46)	73.15 (14.23)	50.93 (10.64)

Note. All cell sizes were 6. Standard deviations are given in parentheses.

To test the hypotheses, MANOVA method for repeated measures analysis (O'Brien & Kaiser, 1985) was performed. In this analysis, sleep deprivation (RSD vs. control) and the number of trials conditioned (single trial vs. three trials) were entered as between-subjects factors. Delta trace and delta context were entered as the dependent variables. The results of

this analysis showed a significant overall multivariate effect of sleep deprivation (Wilk's Λ = 0.3, F (2, 19) = 22.59, $p < .001$), of the number of trials conditioned (Wilk's Λ = 0.44, F (2, 19) = 11.92, $p < .001$) and of sleep deprivation x the number of trials conditioned interaction (Wilk's Λ = 0.46, F (2, 19) = 11.32, $p = .001$).

When each of the two dependent variables was considered separately, univariate tests showed a significant effect of sleep deprivation on both delta trace (F (1, 20) = 32.75, $p < .001$) and delta context (F (1, 20) = 22.68, $p < .001$). This indicated that the RSD rats had fewer delta trace and delta context scores (M = 19.44, SD = 3.72; M = 14.58, SD = 2.58, respectively) than the control rats (M = 49.54, SD = 3.72; M = 31.94, SD = 2.58, respectively). Additionally, univariate tests revealed a significant effect of the number of trials conditioned on both delta trace (F (1, 20) = 14.33, $p = .001$) and delta context (F (1, 20) = 15.02, $p = .001$). This indicated that the rats conditioned in a single trial had fewer delta trace and delta context scores (M = 24.54, SD = 3.72; M = 16.2, SD = 2.58, respectively) than those conditioned in three trials (M = 44.45, SD = 3.72; M = 30.32, SD = 2.58, respectively).

Univariate tests also yielded a significant effect of sleep deprivation x the number of trials conditioned interaction on both delta trace (F (1, 20) = 11.79, $p = .003$) and delta context (F (1, 20) = 16.02, $p = .001$). Simple effect analyses with Bonferroni adjustment were conducted to investigate this interaction further. Contrary to initial analyses, a multivariate simple effect of sleep deprivation in the single-trial condition became non-significant (Wilk's Λ = 0.88, F (2, 19) = 1.28, $p = .30$). Nonetheless, there was a significant multivariate simple effect of sleep deprivation in the three-trial condition (Wilk's Λ = 0.23, F (2, 19) = 32.63, $p < .001$). Simple univariate tests showed a significant effect of sleep deprivation in the three-trial condition on both delta trace (F (1, 20) = 41.97, $p < .001$) and delta context (F (1, 20) = 38.41, $p < .001$). Taken together, the simple effect analyses suggested that, in the single-trial condition, the RSD rats did not significantly differ at delta trace or delta context scores from

the control rats; however, in the three-trial condition, the RSD rats had fewer delta trace and delta context scores than the control rats (see Figure 3 & 4).

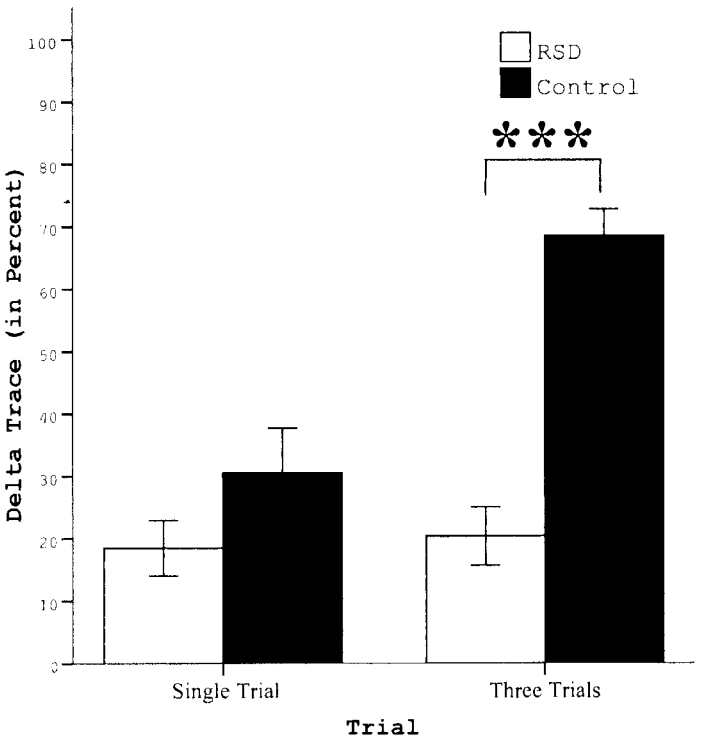

Figure 3. Mean delta trace (in percent; ± SE) by sleep deprivation and by the number of trials conditioned, computed by subtracting percent freezing in the pre-CS period from that in the post-CS period. ***, $p < .001$.

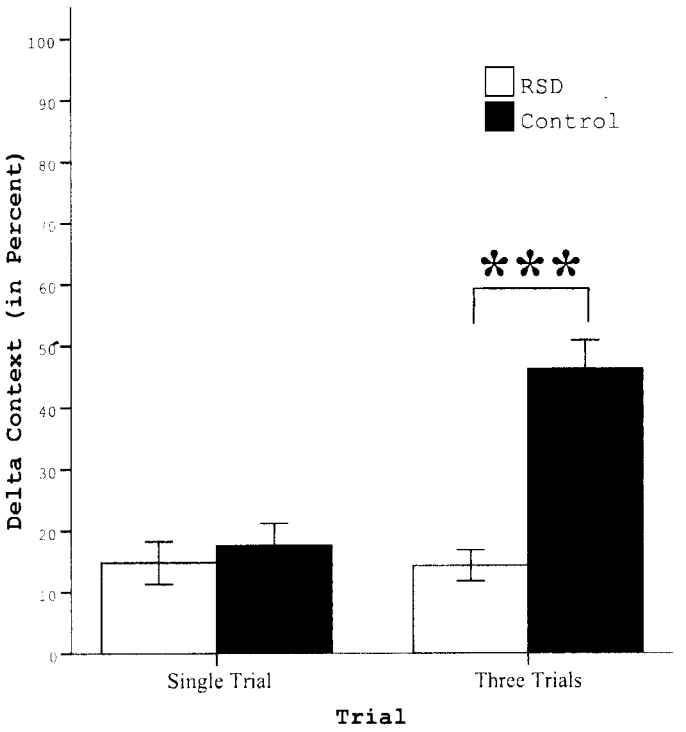

Figure 4. Mean delta context (in percent; ± SE) by sleep deprivation and by the number of trials conditioned, computed by subtracting percent freezing in the pre-CS period from that in the context period. ***, $p < .001$.

Discussion

This study was designed to examine the role of post-training RSD on retention of hippocampus-dependent fear memory including Pavlovian trace and contextual fear memory. Overall, post-training RSD was found to impair retention of hippocampus-dependent memory as the RSD rats had weaker trace and contextual fear memory than the control rats, as indexed by delta scores. Although this did not conform to what Silvestri (2005) found in post-training RSD, it was consistent with what Graves et al. (2003) found in post-training TSD. Therefore, this result supported that post-training RSD has an effect on retention of hippocampus-dependent fear memory by interfering its consolidation process, but failed to support the notion that post-training RSD has no effect on retention of fear memory of any paradigms. In fact, this finding was in line not only with other studies that utilized Pavlovian fear conditioning to investigate the effect of pre-training TSD (Ruskin et al., 2004) and pre-training RSD (McDermott et al., 2003), but also with studies that utilized the Morris swimming maze to investigate the effect of post-training RSD (Smith and Rose, 1996) and pre-training TSD (Guan et al., 2004). Altogether, these behavioral data supported the concept of SD impairment in hippocampus-dependent learning and memory.

Impairment in retention of both trace and contextual fear memory also demonstrated that the effect of post-training RSD on retention did not restrict to spatial modality, required in tasks like contextual conditioning and the invisible-platform version of the Morris swimming maze; it also affected learning tasks requiring auditory modality, like trace conditioning. Previous studies that investigated the effect of SD on hippocampal-mediated memory (e.g., Graves et al., 2003; Ruskin et al., 2004; McDermott et al., 2003; Silvestri, 2005; Fu et al., 2007) often made a comparison between two learning tasks which one required spatial modality and the other did not (e.g., contextual conditioning vs. single cued conditioning, respectively and invisible-platform vs. visible-platform versions of the Morris swimming maze, respectively). Impairment in trace memory shown in the present experiment ruled out

the possibility that the difference in the comparison found in these previous studies was simply due to the difference in modalities of CSs involved between the two tasks and supported that the difference in this comparison stemmed from the effect of SD on hippocampal modulation. Note that, the question to whether the effect of SD is not modality-specific needs further investigation. Future research could be done by studying the effect of SD on learning tasks that require other modalities including olfaction, vision, gestation and tactition.

As a relationship between REM sleep and consolidation of hippocampus-dependent memory is robust, inhibiting REM sleep after acquisition of the hippocampus-dependent task could lead to disturbance in memory consolidation process of the RSD rats. Thereupon, the RSD rats' short-term memory encoded from the task, in turn, might not be able to be transformed to long-term memory, and this might be accounted for poor performance found in the retention testing phrase. In cellular and molecular viewpoint, this RSD disturbance in memory consolidation might reflect an alteration in long-term potentiation (LTP), a cellular model widely accepted to underline memory formation in mammals (Bliss & Collingridge, 1993), in the hippocampus. LTP can be defined by an increase in synaptic strength and in chances of the cells firing action potential in reaction to a constant synaptic input (Sweatt, 2003). It has been previously found in vitro (McDermott et al., 2003) and in vivo (Kim, Mahmoud & Grover, 2005) that RSD disrupted LTP in the hippocampal CA1 neurons. This LTP impairment in response to RSD is probably due to depletion of cAMP-dependent protein kinase (PKA) and protein protein kinase C (PKC). This is because: (1) activation of these kinases by phosphrylation was found to falicitate LTP maintenance (Abel & Lattel, 2001; Ramakers et al., 2002; Thomas, Laroche, Errington, Bliss & Hunt, 1994), (2) inhibition of these kinases by injecting pharmacological inhibitors (e.g., Rp-cAMPs or H7-dihydrochloride) or by using transgenic animals (e.g., R(AB) transgenic mice) resulted in impairment in contextual fear memory (Bourtchouladze et al., 1998; Wallenstein, Vago & Walberer, 2002), (3) the time windows that these inhibitors are effective were found to correspond to specific

time windows for RSD, namely immediately after the acquisition and four hours later (Graves et al., 2003). In short, RSD might operate through the inhibition of some molecular pathways that modulate these protein kinases signaling (Graves et al., 2001).

There are marked alterations in cholinergic and serotonergic systems occurring in the hippocampus during REM, compared to during NREM and awake, that lead to an increase in ACh and a decrease in 5-HT, respectively. SD, in fact, has been found to alter the function of these systems (Youngboold, Smagin, Elkins, Ryan, & Harris, 1999; Porkka-Heiskanen et al., 1995). During REM, this increase in ACh in the hippocampus can enhance the protein kinases by activating M_1 and M_4 muscarinic receptors, and hence, can stimulate LTP (Graves et al., 2001; Migeon & Nathanson, 1994; Dittman et al., 1994). Rudy (1996) found that, when delivered scopolamine, a muscarinic antagonist, rats' contextual fear memory was impaired. Moreover, a facilitative role of ACh to LTP has been confirmed by electrophysiological studies that found an emergence of theta rhythm, the frequencies of neuronal activities, bringing about LTP, when applying ACh to hippocampal slides (Huerta & Lisman, 1995).

Contrary to ACh, decreased levels of 5-HT in the hippocampus during REM is thought to be beneficial to memory formation (Graves, Pack & Abel; Park, 1999). Indeed, SD was found to increase 5-HT turn over (Asikainen et al., 1997). 5-HT can activate the serotonergic receptors, including 5-HT_{1A} and 5-HT_{1B}, resulting in inhibition of adenylyl cyclase (AC), another prominent neural substance positively relating to LTP (Barnes & Sharp, 1999). Hence, the decrease in 5-HT during REM might reduce inhibition of AC activity. Phamarcological studies have shown that 8-HO-DPAT, a 5-HT_{1A} agonist, has retrograde amnesia effects, whereas NAN-190, a 5-HT_{1A} antagonist, facilitates memory (Bevilaqua, et al., 1997; Izquierdo, 1998). Genetic studies confirmed the inhibiting role of 5-HT by showing that 5-HT receptor knockout mice increased REM sleep and better performance in the hidden platform version of the Morris swimming maze (Boutrel et al., 1999; Mallerert et al., 1999). Altogether, ACh and 5-HT were among neuronal substance candidates that might underline amnestic effects of RSD

found in the current study. RSD might alter normal levels of these two substances during REM, probably resulting in improper protein kinases signaling and LTP that, in turn, might lead to impairment in memory consolidation and retention, accordingly. Further studies could examine this possibility further by injecting muscarinic angonist (relating to ACh) and serotonergic antagonist (relating to 5-HT) that would activate during specific time windows of RSD. If improper levels of ACh and 5-HT as a result of RSD are indeed bring about impairment in memory retention, injected RSD animals should have better memory than non-injected RSD animals.

As predicted, overall, the rats trained in the single-trial conditioning had weaker trace and contextual fear memory than those trained in the three-trial conditioning. This might be because it was harder to consolidate memory of a weaker learning task (i.e., single-trial conditioning) than a stronger learning task (i.e., three-trial conditioning). As a manipulation check, it was successfully demonstrated that, in this experiment, different numbers of trials used led to different levels of the strength of learning tasks. That is, the single-trial conditioning was, indeed, a weaker learning task compared with the three-trial conditioning.

As predicted, there was significant interaction between sleep deprivation and the number of trials conditioned. Effects of post-training RSD on retention of hippocampus-dependent fear memory seemed to be mediated by the strength of learning tasks. Further investigation of this interaction revealed that post-training RSD impaired both trace and contextual fear memory only in the three-trial condition, but not in the single-trial condition. This finding, however, did not completely conform to what Graves et al. (2003) found with mice. They showed that contextual memory of mice that were trained in the single-trial conditioning was significantly impaired by post-training TSD. For this reason, it seemed that post-training TSD could successfully weaken fear memory acquired from the single-trial conditioning, but post-training RSD could not. In essence, for post-training RSD to have a noticeable effect on hippocampus-dependent fear memory, it required at least three trials of

fear conditioning. To test this proposition, future investigators could add another group that is sleep deprived by the TSD method. Nonetheless, the discrepancy between the effect of TSD and RSD on single-trial conditioning memory appeared to be perplexing when considering the strong association between REM and memory consolidation. That is, RSD and TSD should disrupt memory consolidation in a similar way since both of them deprive REM sleep. There were at least two possible explanations accounting for this.

The first possible explanation was owing to the stress to which the control rats were faced including changes in humidity and luminosity. It has previously been demonstrated that the stress in this condition selectively impaired hippocampus-dependent fear memory including contextual memory, albeit not as severe as RSD (Dametto et al., 2002). Moreover, stressful situations have been found to interfere with LTP in the hippocampus (Foy, Stanton, Levine & Thompson, 1987; Meseles, Fleshner, Heman, Rose & Diamond, 1999;Pavlides, Nivon, McEwen, 2002) Because of these, even thought hippocampus-dependent fear memory in the control rats was not affected by RSD, it was still attenuated by the stress. Compared to the control condition in Graves' et al. (2003) work where the mice were left undisturbed in their home cages, the control condition in this study had more stress. Moreover, as a manipulation check of the strength of learning showed that the single-trial conditioning was a weak learning task, which might lead to weaker memory formation, impact of the stress in the single-trial condition might attenuate this already-wake formation of fear memory in the control rats to be as low as that in the RSD rats. By contrast, as a manipulation check of the strength of learning showed that the three-trial conditioning was a stronger learning task, which might lead to more robust memory formation, the stress in the three-trial condition could not completely diminish the fear memory in the control rats. As a result, in the three-trial condition, the control rats' fear memory was still in stronger formation than that of the RSD rats. Future researchers could test this possible explanation simply by adding another control group that has no stress involved (e.g., returning them to their home cages). Indeed, since the stress could be regarded as one of

the confounding factors of the present study, by doing so this potential limitation could be eliminated. That is, if the pattern of the results found was really due to the stress, the non-stress control group should exhibit more fear memory than the normal control and the RSD groups.

The second possible explanation was that an ability to learn hippocampus-dependent fear conditioning from a single trial was probably a species-specific ability for mice, not for rats. A failure to find significant post-training RSD impairment in the single-trial conditioning might reflect a flooring effect. That is, in the present experiment, the single-trial condition might be too weak for rats to learn, and hence, all rats that were trained in the single-trial condition (i.e., both RSD and control rats) had only weak memory formation. Post-training RSD, in turn, could not have much impairment on this already-weak memory formation. By contrast, in a study of Graves et al. (2003), it was possible that mice might able to form sufficient fear memory from the single-trial conditioning, and therefore, the effect of SD was more noticeable. Indeed, similar results to Graves' et al. work were found in this study in the rats trained in the three-trial conditioning, which more robust memory formation was allowed to form, compared to the single-trial conditioning. Addition to these two possible explanations, their combination could be regarded as another possible explanation. The stress from the flowerpot technique environment could diminish fear memory of the control rats trained in the single-trial conditioning because this conditioning did not allow these rats to form strong formation of fear memory. On the contrary, the stress from the flowerpot technique environment could not diminish fear memory of the control rats trained in the three-trial conditioning because this conditioning allowed these rats to form stronger formation of fear memory.

It is worthy to discuss the reason why the effect of post-training RSD on retention for hippocampus-dependent memory shown in our experiment was not consistent with what Silvestri (2005) found. In her work, post-training RSD did not interfere with retention of contextual fear memory learned from ten-trial conditioning. The discrepancy between her and

our finding might be due to the strength of the learning tasks. A too-strong learning task might result in a ceiling effect. That is, ten-trial conditioning might lead to too robust fear memory formation, and thus, did not allow the RSD to have much impairment. By combining Silvestri's and our finding together, it could be seen how the strength of learning tasks moderated the effect of post-training RSD on the hippocampus-dependent fear memory. It appeared that post-training RSD selectively affected the hippocampus-dependent fear memory of a somewhat moderate-strength task (i.e., three-trial conditioning). It did not affect fear memory conditioned from a too-weak task (i.e., single-trial conditioning) nor from a too-strong task (i.e., ten-trial conditioning) which might reflect a flooring and a ceiling effect, respectively. Further studies should examine this point further by adding the different numbers of trials conditioned (e.g., two and four till nine). By doing so, the curve of the post-training RSD effect by the strength of the learning tasks could be developed, and the decrement of the post-training RSD effect could be seen, accordingly.

In summary, since both trace and contextual fear memory of the RSD rats were weaker than those of the control rats, post-training RSD appeared to impair retention of hippocampus-dependent memory. Although this did not support idea found in a work of Silvestri (2005) that post-training RSD had no effect on retention of fear memory of any paradigms, the broader concept of SD impairment in hippocampus-dependent learning and memory was supported. The results also extended previous findings by showing that the retrograde amnesia effect of RSD did not restrict to fear memory of cue with spatial modality (i.e., contextual memory); its effect could also be applied to that with auditory modality (i.e., trace memory). Several neural substances, including protein kinases, ACh and 5-HT, were proposed to underline this retrograde amnesia effect of RSD. Moreover, our results along with those of Silvestri (2005) suggested that the effect of post-training RSD was moderated by the strength of learning. The post-training RSD effect was noticeable only with a moderate strength learning task, but not with too weak, nor too strength learning tasks. Note that, imperceptible effect of post-training

RSD found in the weak learning task condition might stem from the stress and the species-specific learning ability of rats.

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