

Effects of β -Estradiol on Enhanced Conditioned Fear Induced by Single Prolonged Stress and Shock in Rats

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ABSTRACT

Introduction: This study examined the effects of administration of subcutaneous β -estradiol on PTSD-like symptoms (the enhanced conditioned fear response, CFR) that induced by a single-prolonged stress (SPS) and shock in rats.

Methods: Adult male Wistar rats were exposed to SPS procedure: restraint for 2 h, forced swim for 20 min, and ether anesthesia. Then the rats were placed in fear conditioning system and received 1 mA electric foot shock for 4 s. Following, stressed rats injected with β -estradiol (600 μ g/kg) or sesame oil. For CFR testing, 24 h later animals were re-exposed to the shock chamber for 3-min without further shock application. Percent of freezing was scored. Following testing, the animals were anesthetized and their brains were removed for histological examination (cell count) of the hippocampus that stained with cresyl violet.

Results: Our results indicated that rats who received electric shock after the SPS exhibited the CFR. β -estradiol significantly reduced the CFR in the SPS rats as compared with control rats. No significant differences were found in cell count in different regions of the hippocampus between experimental groups.

Discussion: Our findings indicated that β -estradiol administration after SPS prevents the enhanced CFR in an animal model of PTSD, suggesting a possible role for β -estradiol in the prevention of PTSD.

1. Introduction

Post-traumatic stress disorder (PTSD) is a psychiatric disorder, which can occur following the experience or witnessing of life-threatening events such as terrorist incidents, military combat, natural disasters, serious accidents, or violent personal assaults. Symptoms of PTSD may include reliving the experience through nightmares and flashbacks, having difficulty sleeping, and feeling detached or estranged (Breslau, Davis,

Andreski, & Peterson, 1991). Cognitive deficits and memory dysfunctions also frequently occur during the development of PTSD (Charles et al., 2006; Leskin & White, 2007; Rauch et al., 2009). Research in basic science and functional neuroimaging has helped to identify three brain regions that may be involved in the pathophysiology of PTSD: the amygdala, medial prefrontal cortex, and hippocampus (Davis & Whalen, 2001). The hippocampus is involved in explicit memory processes and in the encoding of context during fear conditioning (Corcoran & Maren, 2001). Recently, structural neu-

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roimaging studies showed that hippocampal volumes were relatively low in PTSD patients (Li, Han, Liu, & Shi, 2010). It is well established that physiological and behavioral changes observed in animals exposed to single prolonged stress (SPS) could appropriately represent pathophysiological process and core symptomatology of PTSD, including anxiety behavior and cognitive impairments (Iwamoto, Morinobu, Takahashi, & Yamawaki, 2007; Liebsch, Montkowski, Holsboer, & Landgraf, 1998; Takahashi, Morinobu, Iwamoto, & Yamawaki, 2006). SPS paradigms have been extensively applied in the investigation of PTSD. Recent study has further shown that inescapable electric foot shock (S) added to conventional SPS procedures significantly enhanced conditioned and sensitized fear responses (Wang et al., 2008).

The gonadal steroid β -estradiol_ an important mediator of sexual differentiation of the developing brain (Arnold & Gorski, 1984)_ is a potent neuro-protective agent in adult models of brain injury. Estrogen has been associated with a decreased risk, delayed onset and progression, or enhanced recovery from numerous traumatic or chronic neurological and mental diseases, such as abnormal development (dyslexia, autism), abnormal neurotransmitter systems (depression; anorexia/bulimia), disorders caused by trauma (stroke, epilepsy, head injury), abnormal immune (multiple sclerosis) or cardiovascular (stroke, head injury) function (Garcia-Segura, Azcoitia, & DonCarlos, 2001). Numerous studies in rodents have demonstrated the beneficial effects of the β -estradiol, on memory and hippocampal function (Behl, Wildman, Trapp, & Holmberg, 1995; Xiao et al., 2010). One of the hippocampal dependent behaviors that increase in PTSD patients is conditioned fear response. So this study is an attempt to examine this response after SPS & S procedure and then evaluate the effects of administration of subcutaneous β -estradiol in order to prevent PTSD induction.

2. Methods

2.1 Animals

Adult male Wistar rats (200–300 g) were used in this study. Animals were housed five rats in a cage and maintained on a 12-h light/dark cycle. Food and water were provided ad libitum. Behavioral tasks were performed during the light phase of the cycle. All procedures were conducted in agreement with the National Institutes of Health Guide for care and use of laboratory animals.

2.2 Behavioral Procedures

SPS & S: Detailed procedure has been described in previous studies (Liberzon, Krstov, & Young, 1997; Wang et al., 2008). 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 being placed inside a disposable clear polyethylene cone box with only the tail protruding. One end of the cone was closed with tape at the base of the tail. The box size was adjusted according to the size of the rat in order to achieve complete immobilization. A hole in the other end of the cone allowed the rats to breathe freely.

Immediately after immobilization, they were individually placed in a clear acrylic cylinder (240 mm diameter, 500 mm height), filled approximately two-thirds from the bottom with water (24 °C) and forced to swim for 20 min. After 15 min of recuperation, animals were exposed to diethyl ether until they lost consciousness, and then moved into a shock chamber. When they recovered (about 30 min), a single electric foot shock (1 mA for 4 s) was delivered via metal grids installed in the bottom of the chamber. Stressed rats remained in the shock chamber for another 60 s before being returned to the home cages. 24 hours later, animals were re-exposed to the shock chamber for 3-min without further shock. Percentage of freezing (absence of all movement except respiration) was scored.

2.3 Histological Methods

After behavioral testing, rats were anesthetized with a mixture of ketamine (100 mg/kg) - xylazine (4 mg/kg) and perfused intracardially with 0.1 M phosphate buffer for 10 min followed by phosphate-buffered 4% paraformaldehyde for 15 min.

2.3.1 Infiltration and Embedding

The brains were removed and divided at the midline; the right hemisphere was cut in the coronal plane rostral to be septum later. The hippocampus was dehydrated through a graded series of alcohols (50%, 60%, 70%, 80% for 1 h each, 90% and 96% for 1.5 h each and twice 100% for 1.5 h) prior to infiltration. After dehydration, clearing and impregnating of the hippocampal blocks were embedded in disposable tissue molds (Bancroft & Gamble, 2002; McGoey, Reynolds, & Brien, 2003; Trana & Kelly, 2003).

2.3.2 Staining

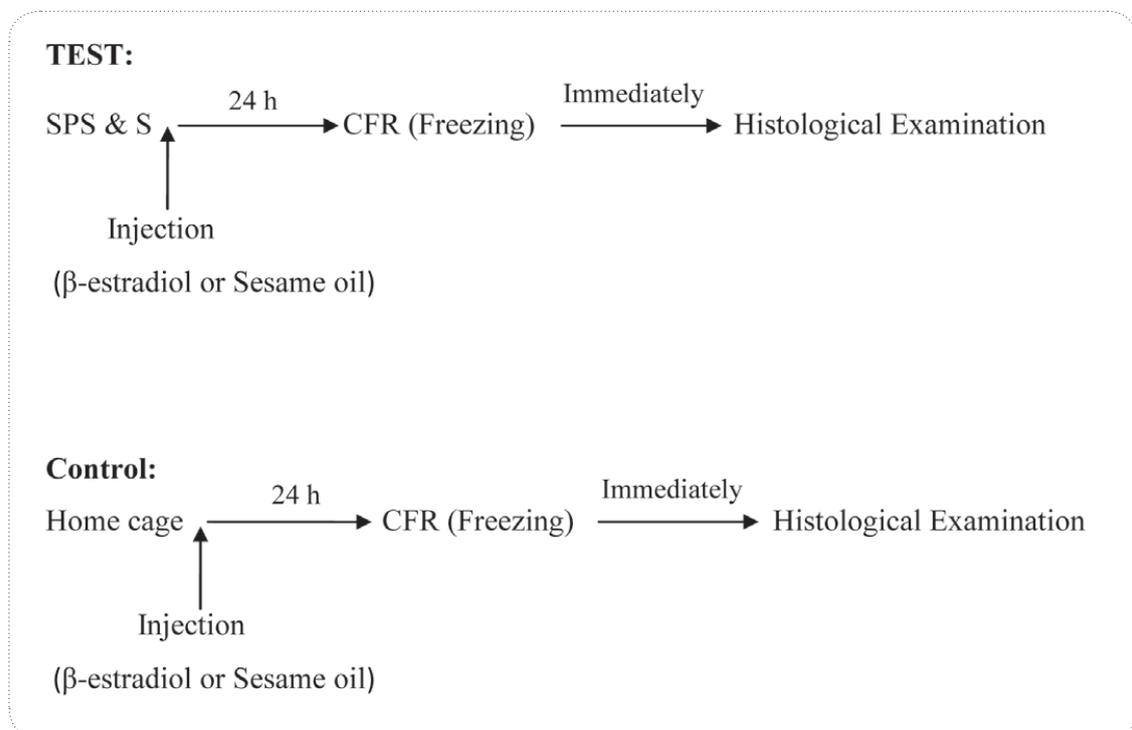
Five coronal sections (7 μ m) from each animal were cut at the level of the dorsal hippocampus and stained by cresyl violet (n=8 in each group). The staining solution contained 0.5 g cresyl violet water solved in 100 ml distilled water. The mounted sections were placed in the staining solution for 20-30 min at room temperature and differentiate in 0.25% acetic acid until most of the stain has been removed (4 - 8 sec) and then briefly pass through absolute alcohol into xylene and check microscopically. If it was necessary differentiation can be repeated. Then sections cleared with xylene and cover slipped using Entellan. Number of pyramidal cells in a 130 μ m segment of each of the hippocampal CA1 and CA3 fields and granule cells in the dentate gyrus (DG) were counted using light microscopy at 400 \times magnifications (Bancroft & Gamble, 2002; McGoey et al., 2003; Trana & Kelly, 2003).

2.3.3 Definitions of Hippocampal Cell Layers

The principal neurons in the different subdivisions of the hippocampus were clearly differentiated from each other. Neurons were counted based on identification of a

clear and distinct nuclear membrane, and counting was restricted to the right hippocampal formation. The pyramidal cell layers and granule cell layer also contain basket cells and glia. Glial cells were easily identified and excluded from the counts, but the basket cells were included in the estimates because their nuclei are similar to pyramidal cells appearance in their respective layers; basket cells comprise less than 1% of the neurons in these layers.

The cell bodies of CA3 are large, elongated and tightly packed in a layer four to five cells deep. The cell bodies and nuclei of the pyramidal cells of CA1 are smaller than those of CA3. At the area near CA1, the transition zone between CA1 and CA3, the cells are also tightly packed in a layer four to five cells deep. Toward the subiculum, the layer becomes progressively loosely packed, and the border with the subiculum is defined as the point at which cells of CA1 cease to be contiguous. The granular layer of the DG contains the smallest and most densely packed cell bodies in the hippocampus. The cell bodies are packed 8–15 cells deep and have well defined borders, and the layer is not in immediate contact with other densely packed layers (Bancroft & Gamble, 2002; McGoey et al., 2003; Trana & Kelly, 2003). The num-



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Figure 1. Timeline of experiment. Test group: Single prolonged stress that immediately followed by an electric foot shock; Control group: Rats received neither aversive SPS procedure nor electric shock. CFR: Conditioned fear response.

ber of survived neurons from three to four sections per animal at dorsal hippocampal level was counted by a blinded observer using a light microscopy, and the neurons as a whole with visible nucleus were counted. The data were expressed as surviving cell number per mm in each regions of the hippocampus (Bancroft & Gamble, 2002; McGoey et al., 2003; Trana & Kelly, 2003).

2.4. Experimental Groups

Rats were randomly divided into the following groups (n = 8 in each group). (A) Control groups: rats removed from their home cage and did not receive aversive stress at the beginning of experiment, and then received sesame oil or β -estradiol subcutaneously. (B) Test groups: rats were exposed to SPS & S according to procedures described in section 2.2. Immediately following shock, the animals received sesame oil or β -estradiol (600 μ g/kg) subcutaneously. One day later, all animals were re-exposed to fear context and the time spent in frozen position was recorded. Percent of CFR (freezing) recorded during 3 min re-exposure (Fig. 1). After the behavioral testing, the histological examination was done according to procedures described above.

2.5. Statistical Analysis

One-way analysis of variance (ANOVA) was used for comparing the groups. When analysis of variance showed a significant difference, post hoc Tukey HSD test were applied to demonstrate the difference. In each

test, the data are expressed as the mean \pm SEM and $P < 0.05$ is accepted as statistically significant.

3. Results

3.1. Behavioral Testing Results

One way ANOVA on freezing data showed a significant effect of β -estradiol ($F_{3,28} = 368.17$, $P = 0.000$). Post-hoc comparison indicated that the percent of freezing in SPS & S + sesame oil group was significantly higher than control + sesame oil ($P = 0.000$). SPS rats given a single high dose of β -estradiol showed decreased the percent of freezing as compared with SPS rats that received sesame oil ($P = 0.0001$). So we have found that the percentage of freezing in SPS & S + sesame oil group was significantly increased as compared with control + sesame oil, but this increase was reversed by β -estradiol administration. There were no differences between two control groups ($P = 0.778$) and control + β -estradiol and SPS & S + β -estradiol groups ($P = 0.25$) (Fig. 2).

3.2. Histological Examination Results

We assessed cell survival with Nissl staining, in 130- μ m segments of hippocampal main regions. Figure 3 represents the photographs of coronal sections containing the hippocampal regions. The average number of neurons in all sections of each group was shown in table 1. The number of pyramidal cells in DG, CA1, and CA3 showed no significant differences between all groups.

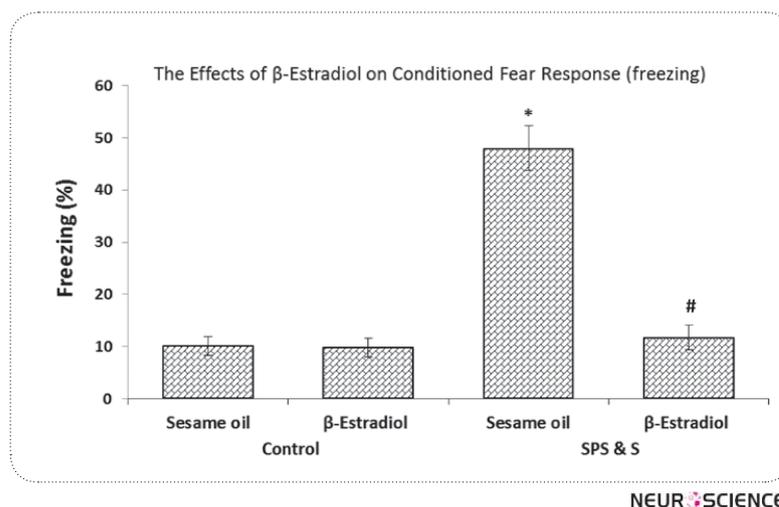


Figure 2. Effects of β -estradiol on conditioned fear response after PTSD induction. * $P = 0.000$, as compared with control + sesame oil group. # $P = 0.0001$ as compared with SPS&S group that received sesame oil. Data are shown as mean \pm SEM (n = 8).

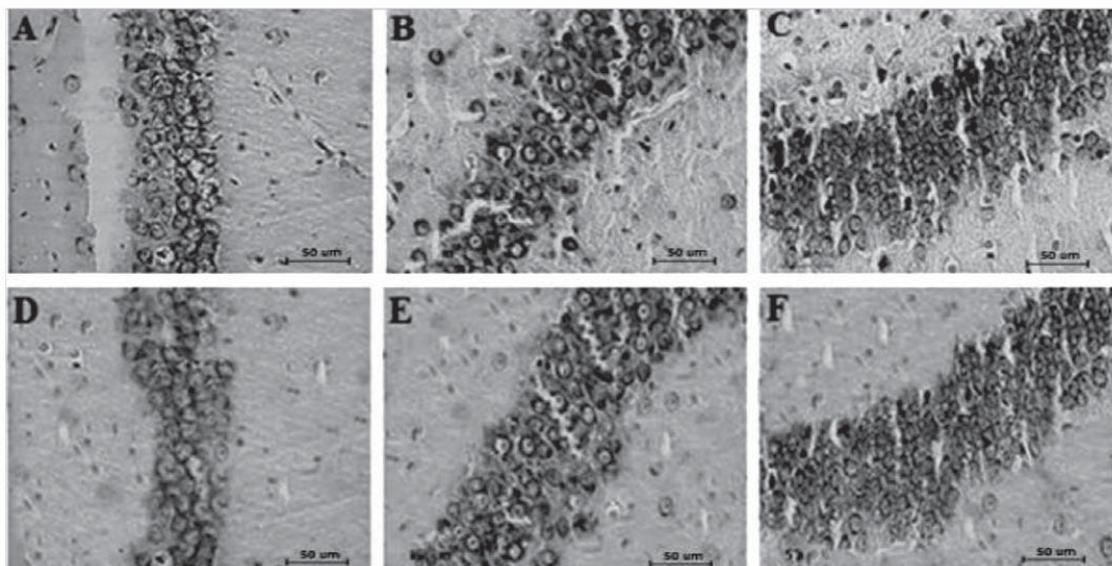
4. Discussion

The main purpose of the present study was to investigate the effects of subcutaneous injection of β -estradiol following SPS & S training on subsequent CFR in male rats. We have demonstrated that β -estradiol reduces the elevated CFR induced by SPS & S paradigm. Rats receiving β -estradiol demonstrated shorter freezing levels during the contextual test.

A rat model involving SPS has been developed and employed for PTSD research (Iwamoto et al., 2007; Khan & Liberzon, 2004). In this study, the SPS model was modified by giving rats a single inescapable electric foot shock after SPS procedure (SPS & S) and the CFR was measured. There are many reports that CFR was enhanced in PTSD animals (Wen et al., 2008). Our data demonstrated that this model produces a core symptom of PTSD, the enhanced fear response to the traumatic cue. The hippocampus is widely believed to be essential for learning about the context in which conditioning occurs. Estradiol influences hippocampus-dependent contextual fear conditioning, but not hippocampus-independent cued fear conditioning (Gupta et al., 2001; Markus & Zecevic, 1997). Physiologically high levels of estradiol influence contextual fear conditioning negatively, as female rats in pro-estrus or treatment with a high dose of estradiol reduces the amount of freezing after conditioning as compared with females in estrus (Altemus, Conrad, Dolan, & McEwen, 1998; Gupta et

al., 2001; Markus & Zecevic, 1997), whereas ovariectomy increases freezing behavior in response to a context associated with shock (Gupta et al., 2001). Numerous studies in rodents have demonstrated the beneficial effects of the most potent estrogen, β -estradiol, on memory and hippocampal function (Behl et al., 1995). The results from this study showed that β -estradiol influence the hippocampus-dependent CFR. Our data demonstrated that β -estradiol exposure significantly decreases CFR after PTSD induction. This finding suggests that β -estradiol is able to prevent development of PTSD symptoms in rats.

Several lines of evidence have demonstrated a strong relationship between atrophy of the hippocampus and PTSD. Magnetic resonance imaging volumetric studies have consistently reported decreased hippocampal volumes in PTSD (Hedges et al., 2003). In a meta-analysis, there was significantly smaller volume in both right and left hippocampus in adult subjects with chronic PTSD as compared with both healthy controls and acutely traumatized controls (Kitayama, Vaccarino, Weiss, & Bremner, 2005). Base on previous observations, apoptosis might be involved in hippocampus atrophy in PTSD (Yan, Liu, & Li, 2006; Yao, Tan, Gu, Ye, & Wang, 2007). Estrogens are known as potent regulators of brain functions including proliferation, survival and plasticity, (McEwen, 2001) and have been shown to alter the density of pyramidal cells in the hippocampus (Kitayama et al., 2005). β -estradiol attenuates neural



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Figure 3. Nissl staining of hippocampal cells. A, B, and C: SPS&S group that received sesame; D, E, and F: SPS&S group that received β -estradiol. A, D: CA1; B, E: CA 3; C, F: DG.

Table 1. The effects of SPS&S and β -estradiol administration on the neural cell count in different regions of the hippocampus. The average number of neurons in five coronal sections (7 μ m) from each animal (n = 8). Number of cells in a 130 μ m segment of each of the hippocampal fields was counted using light microscopy at 400 X magnifications. Data are expressed as means \pm SEM.

Group	Number of Cells in Hippocampal Regions		
	CA1	CA3	DG
Control (Sesame oil)	21.12 \pm 1.45	22.25 \pm 2.6	33.37 \pm 3.15
Control (β -estradiol)	24.10 \pm 2.94	24.00 \pm 2.92	31.12 \pm 2.69
SPS&S (Sesame oil)	24.75 \pm 1.64	25.00 \pm 2.97	33.62 \pm 3.10
SPS&S (β -estradiol)	22.5 \pm 1.06	22.37 \pm 1.68	32.12 \pm 2.53

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injury due to stroke, seizures, excitotoxicity, oxygen-glucose deprivation and oxidative stress.

Since estradiol's effects on behavior may be linked to its ability to alter the number of cells in the hippocampus, another aim of this research was to identify potential neuro-protective effects of β -estradiol that can ameliorate PTSD-induced CNS damage. We evaluated cell density in all sub-fields of the hippocampus. Our data demonstrated that the number of cells showed no differences in all regions of the hippocampus between control and SPS & S groups. One possibility is the lower sensitivity of cresyl violet staining as compared with other staining assay such as TUNEL assay. Thus, more sensitive assay must be used to determine the exact number of apoptotic cells. Another possibility is that the CFR was tested 24 hours after SPS&S exposure and estradiol administration. Although, this time is sufficient to observe the behavioral effects of estradiol, it seems a longer time is needed that SPS&S and estradiol could influence the cell count in different regions of the hippocampus. In conclusion, the findings of the present study demonstrate that β -estradiol administration after SPS prevents the enhanced CFR in an animal model of PTSD, suggesting a possible role for β -estradiol in the prevention of PTSD.

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