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A single prolonged stress paradigm produces enduring impairments in social bonding in monogamous prairie voles

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Abstract

Traumatic events such as natural disasters, violent crimes, tragic accidents, and war, can have devastating impacts on social relationships, including marital partnerships. We developed a single prolonged stress (SPS) paradigm, which consisted of restraint, forced swimming, and ether anesthesia, to establish an animal model relevant to post-traumatic stress disorder. We applied a SPS paradigm to a monogamous rodent, the prairie vole (*Microtus ochrogaster*) in order to determine whether a traumatic event affects the establishment of pair bonds. We did not detect effects of the SPS treatment on anhedonic or anxiety-like behavior. Sham-treated male voles huddled with their partner females, following a 6 day cohabitation, for a longer duration than with a novel female, indicative of a pair bond. In contrast, SPS-treated voles indiscriminately huddled with the novel and partner females. Interestingly, the impairment of pair bonding was rescued by oral administration of paroxetine, a selective serotonin reuptake inhibitor (SSRI), after the SPS treatment. Immunohistochemical analyses revealed that oxytocin immunoreactivity (IR) was significantly decreased in the supraoptic nucleus (SON), but not in the paraventricular nucleus (PVN), 7 days after SPS treatment, and recovered 14 days after SPS treatment. After the presentation of a partner female, oxytocin neurons labeled with Fos IR was significantly increased in SPS-treated voles compared with sham-treated voles regardless of paroxetine administration.

Our results suggest that traumatic events disturb the formation of pair bond possibly through an interaction with the serotonergic system, and that SSRIs are candidates for the treatment of social problems caused by traumatic events. Further, a vole SPS model may be useful for understanding mechanisms underlying the impairment of social bonding by traumatic events.

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Keywords

PTSD; Partner preference; Social behavior; Serotonin SSRI Paroxetine

1. Introduction

The establishment of social relationships with family members, colleagues, and neighbors is critical for health and wellbeing. However, traumatic events sometimes interfere with the ability to form social relationships. Recent reports indicate significant comorbidity between post-traumatic stress disorder (PTSD) and social anxiety [1,2]. Social attachment between sexual partners is one of the basic social relationships that is important for mental health. The pair bond is a selective and enduring relationship between mating partners in social monogamous animals, including human beings [3–5]. Traumatic events deteriorate not only social relationships with friends but also marital relations [6]. Higher levels of post-traumatic stress symptoms are associated with lower couple functioning [7]. Veterans with PTSD exhibit more frequent displays of hostility and fewer expressions of acceptance and humor in both themselves and their partners [8]. The molecular and cellular mechanisms underlying the deterioration of the ability to maintain social relationships by PTSD are still unknown, although functional neuroimaging studies implicate several brain regions including amygdala, ventral medial prefrontal cortex, dorsal anterior cingulate cortex, and hippocampus, in PTSD [9]. An animal model of single prolonged stress (SPS) is useful for studying neurobiological mechanisms of PTSD, because of their construct, face and predict validity [10]. A SPS paradigm is comprised of three stressors in a single day (restraint, forced swimming, and a loss of consciousness by ether anesthesia) and a 7-day quiescent period. The quiescent period is required for PTSD-like phenotype to develop, rather than only a single stressor day [11]. SPS-treated rats mimic neuroendocrinological impairments observed in PTSD patients such as enhanced negative feedback of the hypothalamo-pituitary-adrenal axis [11]. Neuroendocrinological impairments in SPS-treated rats are associated with an increased level of glucocorticoid receptor (GR) mRNA and down-regulation of mineralocorticoid receptor (MR) mRNA in the hippocampal CA1 and CA2 regions [12]. Further, SPS-treated animals also replicate deficits in the extinction of fear memories, which is one of cardinal symptoms in PTSD patients [13,14]. Elevated fear responses caused by a SPS treatment are ameliorated by chronic administration of paroxetine, which is a selective serotonin reuptake inhibitor (SSRI) and is sometimes administrated to PTSD patients [15]. Although there are many studies using animal models of PTSD including the SPS paradigm, there are no reports describing the influences of traumatic events specifically on social attachment behaviors, especially pair bonding, since traditional laboratory animals such as rat (*Rattus norvegicus*) and mouse (*Mus musculus*) do not display a socially monogamous mating strategy. [16–18]

The prairie vole (*Microtus ochrogaster*) is a socially monogamous rodent, which forms a pair bond with its mate, and has contributed significantly to our understanding of the neurobiological mechanisms underlying the formation and maintenance of the pair bond and other prosocial behaviors [4,19,reviewed in 20,21]. Cohabitation with an opposite sex animal for 24 h is enough to produce an enduring pair bond, which is indicated by showing a

partner preference and aggression toward conspecific strangers [22]. Two related neuropeptides, oxytocin and vasopressin, are crucial for the formation and maintenance of pair bond. These neuropeptides are mainly produced in neurons in paraventricular and supraoptic nuclei (PVN and SON, respectively) of hypothalamus, although other brain regions also contain neurons producing them [23–25]. Oxytocin neurons in the PVN and SON modulate behavioral responses associated with pair bonding by releasing oxytocin in the accumbens nucleus (NAcc) [26]. In addition to the NAcc, oxytocin neurons projecting to some brain regions such as the amygdala and olfactory bulb from the PVN are proposed to modulate pair bonding formation [27,28]. In females, and perhaps males as well, oxytocin facilitates pair bond formation through oxytocin receptors at the NAcc and prefrontal cortex, since the injection of oxytocin receptor antagonist during cohabitation prevents pair bond formation [29]. Recently, oxytocin receptor signaling has been reported to be crucial for pair bond formation in male prairie voles, since male prairie voles intracerebroventricularly administered oxytocin receptor antagonist do not show partner preference and show reduced sexual behavior [30]. Furthermore, males with a genetic polymorphism leading to reduced oxytocin receptor expression in the NAcc display impairments in pair bond formation [31]. In males, the fact that the administration of vasopressin 1a receptor antagonist at the lateral septum (LS) and ventral pallidum (VP) disrupts pair bonding indicates that vasopressin 1a receptor at these brain regions is also necessary for pair bond formation and perhaps expression in males [32,33]. Overexpression of vasopressin 1a receptor at the VP using viral vector gene transfer enhances partner preference in promiscuous meadow voles [34]. Involvement of oxytocin receptor at the LS in the pair bond formation is also suggested in male prairie voles [35]. In male prairie voles, oxytocin enhances the correlated connectivity of brain regions involved in social information processing and reward [30].

In addition to neuropeptides, the dopamine system modulates pair bond formation. The activation of dopamine D1 receptor at the NAcc prevents pair bond formation, whereas the activation of D2 receptor facilitates it [36]. Besides the ventral tegmental area, various brain regions contain neuronal cells expressing tyrosine hydroxylase (TH), which is the rate limiting enzyme for synthesis of dopamine and other catecholamines. TH-immunoreactive (IR) cells in the principal bed nucleus of the stria terminalis (pBNST) and medial amygdala (MeA) are suggested to be related to social behaviors across the reproductive cycles [37].

Here we applied a SPS paradigm to male prairie voles in order to determine whether traumatic events impair pair bond formation in this model organism or not. SPS-treated voles did not show a partner preference, the laboratory proxy for a pair bond, although anhedonia and enhancement of anxiety-like behavior was not affected by the SPS paradigm. OxytocinIR in the SON was transiently reduced after the SPS treatment. Interestingly, administration of an SSRI rescued the impairment in the display of partner preference following SPS. It is possible that traumatic events disturb pair bond formation through the perturbation of the coordinated functions of oxytocin and serotonin systems.

2. Materials and methods

2.1. Animals

Prairie voles were housed in a polycarbonate standard cage (32 × 21 × 12 cm) under standard laboratory conditions (a 12 h light/dark cycle, 23°C, bedding with wood shavings (white flake, Oriental Yeast Co., Ltd., Tokyo, Japan), free access to food (standard rabbit chow RC4, Oriental Yeast Co., Ltd.) and water ad libitum). Animals were housed 4–6 per cage in same sex groups after weaning at 4–5 week-old. Sexually naïve male prairie voles (21 ± 3.0 week-old) were used as subjects. Subjects were individually housed for 4 days before the SPS or sham treatment. All animal experiments were performed in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan, and approved by the Animal Experiment Committee at Gunma University.

2.2. SPS treatment

SPS treatment was performed according to a previous report [11] with a modification. After 4 days of individual housing, experimental subjects were restrained within a 50 ml conical tube that had holes for ventilation for 2 h, and were forced to swim in water at 23° for 5 min. We determined the duration of the forced swimming in preliminary experiments so that subject voles did not drown, although the forced swimming was originally applied for 20 min in the rat SPS paradigm. After recuperation for 15 min in their home cage, they were anesthetized by diethyl ether until they lost consciousness. They were returned to their home cage and were undisturbed for 7 days under an individual housing condition. We defined the day when SPS treatment was performed as the Day 0. Prairie voles handled for 20–30 s in their cage were used as controls (sham treatment). Sham-treated voles were individually housed from the Day –4 until they were cohabited with a female vole.

2.3. Behavioral and immunohistochemical analyses

2.3.1. Experiment 1: Effect of SPS treatment on anxiety, sucrose preference, and pair bonding—In the first experiment, effects of SPS on anxiety, anhedonia, and pair bonding were investigated in experimental and sham male prairie voles. Behavioral tests were performed at a corner partitioned with curtains in the same room that the SPS treatment was performed. A schematic illustrating the experimental procedure is presented in Fig. 1A. Subjects were randomly separated into 2 groups: one is for sham treatment (n = 12) and another is for the SPS treatment (n = 12). They were given 1% sucrose solution for 2 days in order to decrease the variability in sucrose consumption. The position of bottles was exchanged every day to prevent a positional effect. The subjects were given free choice between sucrose solution and tap water for 12 h at the Day 1 and Day 8. No previous food or water deprivation was applied before the test. The consumption of sucrose solution and water was calculated by weighing the bottles. Sucrose preference (%) was calculated as following: weight of sucrose solution intake/(the sum of the weights of sucrose solution and water intake)*100. Sucrose preference was defined as over 65% of sucrose intake. Only prairie voles that showed sucrose preference at Day –1 were analyzed. Four voles were excluded from the analysis because of technical troubles such as leakage of solution. Finally

we analyzed 9 and 10 voles for sham treatment and SPS treatment, respectively. Excluded animals were included in other analyses as possible.

Three voles did not survive the SPS treatment, when it was applied at Day 0 as described above.

At Day 7, an open field test was performed as previously described [38]. Subjects ($n = 12$ for sham treatment, $n = 9$ for SPS treatment) were placed on the center of an open field box ($40 \times 40 \times 40$ cm) with light of 350 lx and allowed to investigate freely for 10 min. The duration subjects spent in the center area (20×20 cm) was measured. The number of fecal boli was also counted.

After the open field test, subject male prairie voles were cohabited with a sexually naïve female prairie vole between the Day 8 and 14. Partner preference was investigated at the Day 14 using a three chamber apparatus according to Lim et al. [34] with some modifications. The apparatus was composed of three acrylic chambers with the same size ($20 \times 25 \times 20$ cm). A center chamber was placed behind left and right chambers which stood by each side. The center chamber was connected to left and right chambers with hollow tubes (6 cm diameter $\times$ 10 cm), so that subjects were free to move in each chamber. Chambers had wooden chip bedding. A partner female, which was cohabited with the subject prairie vole, was tethered to a right or left chamber, and a novel “stranger” female was tethered to the other chamber. A subject prairie vole was placed in the center chamber and allowed to freely move in the apparatus for 3 h. Behaviors were videotaped. The duration of huddling behavior with each female, the duration spent in a left or right chamber, and the number of entries into chambers were measured. Three sham-treated subjects which showed sexual behavior to female voles during the partner preference test were eliminated from the analysis, but included in other analyses. Measurements were performed by an observer who was blind to the treatment group of each subject ($n = 9$ for sham treatment, $n = 9$ for SPS treatment).

2.3.2. Experiment 2: Effect of SSRI administration on SPS-mediated impairment of partner preference—

Subject voles were given the SPS treatment as described above and the experimental paradigm is presented in Fig. 1B ($n = 12$ for SPS treatment and administrated with paroxetine, $n = 12$ for SPS treatment and administrated with the vehicle). Paroxetine (Teva Pharmaceutical Industries LTD., Petach Tikva, Israel) was dissolved in distilled water at 0.05 mg/ml. Paroxetine (10 mg/kg) or water was orally administrated twice a day using feeding needles between the Day 1 and 10 (Fig. 1B). Subjects were cohabited with a female prairie vole for 3 days from the Day 7. In this experiment, we shortened the duration of cohabitation compared with experiment 1, since it is enough duration for the spontaneous formation of pair bonding [20,21]. A partner preference test was carried out at the Day 11. Two voles (one vole for each treatment) were excluded from the analysis because stimulus female voles pinched their forepaws in tethering chain. During the following 3 days, subjects were housed alone. At Day 14, 90 min after their partner voles were presented for 10 min, subjects were deeply anesthetized with avertin and transcardially perfused with phosphate-buffered saline (PBS) followed by 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde and 15.6% saturated picric acid.

Brains were extracted and immersed in 20% sucrose in 0.1 M phosphate buffer (pH 7.4) at 4°C for 2 days.

Brains (n = 11 for SPS treatment and administrated with paroxetine, n = 12 for SPS treatment and administrated with the vehicle, n = 8 for untreated control voles) were sliced at 40 µm using a cryostat (CM3050S, Leica Biosystems GmbH, Nussloch, Germany), collected at 240-µm intervals, and stored in cryoprotectant (0.1 M phosphate buffer, pH 7.4, 0.9% NaCl, 30% sucrose, 1% polyvinylpyrrolidone, 30% ethylene glycol) at -20°C until use. One brain for SPS-treated vole with vehicle administration was eliminated because of inadequate perfusion. According to a previous report [39], immunological staining was performed. Endogenous peroxidase was inactivated by incubating in PBS containing 0.7% H₂O₂ and 40% ethanol for 15 min after washing with PBS three times. Sections were washed with PBS containing 0.3% Triton X-100 (PBS-T) three times, and then incubated with rabbit anti-cFos antibody (SC-52, Santa Cruz Biotechnology Inc., Dallas, TX, 1:500 dilution) in PBS-T containing 1% bovine serum albumin overnight at room temperature. Sections were washed with PBS-T three times, and incubated with biotinylated anti-rabbit IgG antibody (1:1,000 dilution, Vector Laboratories Inc., Burlingame, CA) for 2 h. Sections were incubated with the complex of avidin-peroxidase and biotin (1:1,000 dilution, Vector Laboratories Inc.) for 2 h. Immunoreactivity was visualized by incubating in 0.25 mg/ml diaminobenzidine and 0.01% H₂O₂ in Tris-HCl (pH 7.5). After washing with PBS-T three times, sections were stained using rabbit anti-oxytocin (#20068, ImmunoStar Inc., Hudson, WI, 1: 10,000 dilution) or rabbit anti-vasopressin antibody (AB1565, EMD Millipore Corp., Billerica, MA, 1: 10,000 dilution). Color development was performed using SG Peroxidase Substrate Kit ImmPACT (Vector Laboratories Inc.). Sections were mounted on glass slides and photographed using a microscope (ECLIPS 80i, Nikon Instruments Inc., Tokyo, Japan) combined with a CCD camera (DFC290, Leica Biosystems GmbH). The number of cells double-labeled with cFos as well as oxytocin or vasopressin was manually counted.

2.4. Experiment 3: Immunohistochemical survey for neurochemical alterations caused by the SPS treatment

At Day 7, brains were prepared from subjects of a new cohort (n = 8 for sham treatment, n = 8 for SPS treatment) and the experimental timeline is shown in Fig. 1C.

Immunohistochemistry was performed as above described in order to clarify the effects of SPS treatment on several proteins involved in modulating pair bonding. Primary antibodies used here are following: anti-glucocorticoid receptor (GR, 1:1,000 dilution), anti-mineralocorticoid receptor (MR, 1:1,000 dilution), anti-oxytocin (1: 20,000 dilution), anti-vasopressin (1:10,000 dilution), and anti-TH (GTX13016, GeneTex Inc., Irvine, CA, 1:4,000 dilution). Anti-GR and Anti-MR antibodies were kindly supplied from Dr. Kawata, Kyoto Prefectural University of Medicine (present affiliation, Bukkyo University, Kyoto). Immunoreactivity was visualized using diaminobenzidine as above.

The intensity of IR signals in all specimens containing regions of interest was measured using an ImageJ software (NIH), except for GR and MR IRs whose intensities were measured bilaterally in 3 consecutive sections. The numbers of measured sections are

indicated in Table 2. Measured values were estimated in the entire nuclei of PVN, SON and pBNST, or entire regions of LA and MeA.

2.5. Statistics

All of the statistics were calculated using IBM SPSS statistics software (Version 21.0, IBM Corp., Armonk, NY) after the removal of outliers identified in box plots. Statistical comparisons for immunohistochemical analysis and open field test were made by Student's *t*-test. Sucrose preference test, partner preference test, and IR in paroxetine-administrated voles were compared by ANOVA with Tukey's post hoc tests for pairwise comparison. The data are presented as means $\pm$ SEM. *P* values of less than 0.05 were considered significant.

3. Results

3.1. Experiment 1: Pair bonding was disturbed in SPS-treated voles, but anhedonia and anxiety behaviors were not observed

In the sucrose preference test, all of 9 sham-treated voles and 10 out of 11 SPS-treated voles showed the preference to sucrose-containing water, when $>65\%$ of sucrose uptake against total uptake of liquid was defined as sucrose preference. A two-way ANOVA showed that SPS treatment did not affect the uptake of sucrose-containing water (Fig. 2A, main effect of treatment, $F_{3,31} = 1.405$, $P = 0.245$; timing, $F_{3,31} = 0.812$, $P = 0.375$, the interaction of treatment and timing, $F_{3,31} = 1.362$, $P = 0.252$). In the open field test, there was no difference in the duration that subjects spent at the center area between SPS-treated and sham-treated voles (Fig. 2B, $T_{19} = 0.945$, $P = 0.356$). The number of feces produced by SPS-treated voles was comparable to that of sham-treated voles ($p > 0.05$, data not shown).

In the partner preference test, in terms of huddling time, a two-way ANOVA detected a significant effect of time with stimulus female voles (partner or stranger) and the interaction between treatment and stimulus vole (the main effect of treatment, $F_{3,28} = 0.013$, $P = 0.910$; stimulus, $F_{3,28} = 4.873$, $P = 0.036$; the interaction between treatment and stimulus, $F_{3,28} = 9.069$, $P = 0.005$). Post hoc analysis showed that SPS-treated voles indiscriminately huddled with their partner and stranger voles ($P = 0.548$), whereas sham-treated voles huddled with their partner longer than with stranger female ($P = 0.002$) (Fig. 3A). A two-way ANOVA also detected a significant interaction between treatment and stimulus female voles in the duration that subjects spent in each cage (the main effect of treatment, $F_{3,28} = 0.000$, $P = 1.000$; stimulus, $F_{3,28} = 0.043$, $P = 0.838$; the interaction between treatment and stimulus, $F_{3,28} = 21.847$, $P < 0.001$). Post hoc analysis indicated that SPS-treated voles spent more time in a stranger-containing chamber than a partner-containing chamber ($P = 0.001$), in contrast to sham-treated voles which spent more time in the chamber tethering their partner voles than that tethering stranger voles ($P = 0.006$) (Fig. 3B). There was no significant difference in terms of the number of entries into chambers (the main effect of treatment, $F_{3,28} = 0.055$, $P = 0.817$; stimulus, $F_{3,28} = 2.180$, $P = 0.151$; the interaction between treatment and stimulus, $F_{3,28} = 0.710$, $P = 0.407$). The total number of entries into chambers was also not different between sham- and SPS- treated voles (Sham-treated voles 203.1 ± 27.3 , SPS-treated voles 203.0 ± 21.7 ; $T_{14} = 0.188$, $P = 0.854$).

3.2. Experiment 2: Paroxetine alleviated the impairment in pair bonding produced by SPS

A two-way ANOVA detected significant effect of stimulus female voles (the main effect of treatment, $F_{3,40} = 0.168$, $P = 0.684$; stimulus, $F_{3,40} = 8.452$, $P = 0.006$; but no interaction between treatment and stimulus female was detected, $F_{3,40} = 1.614$, $P = 0.211$). Post hoc analysis again revealed that new cohort of SPS-treated voles lost partner preference ($P = 0.254$), when they were administrated with tap water. On the contrary, when paroxetine was daily administrated after SPS treatment, SPS-treated voles spent longer time huddling with their partners than strangers ($P = 0.005$) (Fig. 4A). In terms of the duration in a chamber, SPS-treated voles administrated with water or paroxetine spent similar duration in chambers tethering the partner and that tethering the stranger female (the main effect of treatment, $F_{3,40} = 0.016$, $P = 0.899$; stimulus $F_{3,40} = 0.101$, $P = 0.752$; the interaction between treatment and stimulus, $F_{3,40} = 1.092$, $P = 0.302$). Huddling is considered a more sensitive measure of partner preference than time in cage [40].

When we measured the intensity of oxytocin IR in SPS-treated voles administrated with paroxetine at the Day 14, there was no difference among treatments (Table 1). We then counted the number of cells labeled with both oxytocin and cFos IRs. Like single staining, oxytocin IR was appeared as blue-gray color at somatic bodies and neurites, and cFos IR was observed as brown at nuclei of cells, suggesting the correct representation of IRs for these antigens (Fig. 5). SPS-treated voles showed increased number of cells double-labeled with oxytocin and cFos IRs in the SON, but not in the PVN, compared to untreated voles, regardless of the administration of paroxetine or tap water (Table 1). There were no differences among treatments in the number of cells dual-labeled with vasopressin and cFos IRs (Table 1, Supplementary Fig. 3).

3.3. Experiment 3: Immunohistochemical analyses of SPS-treated voles

SPS treatment is known to affect the expression of GR and MR in the hippocampus in the rat [12]. Anti- GR and MR antibodies detected the antigens in the hippocampal neurons of prairie voles (Supplementary Fig. 1). However, statistical analysis did not detect significant difference in either MR- or GR-IR between sham-treated and SPS-treated voles (Table 2).

We also investigated the effects of SPS treatment on the expression of molecules that are essential for pair bonding. Vasopressin and oxytocin were detected in neurites and somatic bodies at the hypothalamic regions including the PVN and SON (Fig. 6). The intensity of vasopressin IR was not different between SPS-treated and sham-treated voles in either PVN or SON. In contrast, the intensity of oxytocin IR in the SON was significantly lower in SPS-treated voles than sham-treated voles ($T_{14} = 2.305$, $P = 0.037$, Table 2). Oxytocin IR at the PVN was not different between treatments.

TH, which is a key enzyme for dopamine synthesis, was detected at somatic bodies and axonal fibers in various brain regions (Supplementary Fig. 2). However, statistical analyses did not detect significant difference between treatments in any regions of MeA, PVN, pBNST, or LS. Results of statistical analyses are summarized in Table 2.

4. Discussion

Experimental voles were considered to display a partner preference if the group huddled with partners significantly longer than with the strangers, like sham-treated voles showed (Fig. 3A). The fact that the numbers of entries into chambers was equivalent between the treatments (Fig. 3C) suggests that disturbed partner preference was not due to the impairment of locomotor or exploration activity. Hence, we concluded that SPS treatment disturbed the formation of pair bonding in prairie voles, as inferred by the partner preference, even though subjects were cohabitated with a female vole for 6 days. Further, we replicated this finding using another cohort of voles using a shorter cohabitation period. The display of a partner preference requires 2 temporally distinct processes: pair bond formation as well as its recall, or expression [41]. The administration of vasopressin V1a receptor (V1aR) antagonist at different time points indicates that V1aR signaling is necessary for both the formation and expression of partner preference and that these processes are dissociable [33]. Other neurotransmitter receptors, such as oxytocin receptor in the medial prefrontal cortex, NAcc, and BNST, as well as dopamine D2 receptor in the NAcc, contribute to pair bonding formation, whereas successful mating and pair bonding increases the density of V1aR in the anterior hypothalamus and oxytocin receptor and dopamine D1 receptor in the NAcc [reviewed in 41], suggesting that distinct neurochemical mechanisms modulate the formation and expression of pair bonding, respectively. In this study, cohabitation with a female followed the SPS treatment, suggesting a failure in pair bond formation rather than recall or expression. This experiment cannot dissociate between recall or expression. In Experiment 1, subject voles were cohabited with a female for 6 days, which was 14 days after the SPS-treatment. It is well known that 24 h of cohabitation is enough to the formation of pair bond [22,23], suggesting a long lasting disturbance of the SPS treatment on pair bond formation and/or expression. A previous study revealed that early-life social isolation in the first two weeks of life can also produce long-term impairments in partner preference formation [42]. In contrast, either the stress of forced swimming or the injection of corticosterone facilitates pair bonding [43]. Such discrepancy may be attributed to differences in experimental conditions such as acute and chronic effects of stressors or strength of the given stress. The authors of the above referenced study cohabitate experimental animals for 30 min immediately after the treatment, whereas we paired them 7 days after the SPS treatment. Further, the SPS treatment consists of restraint, forced swimming and anesthesia, in contrast earlier studies apply only forced swimming for 3 min. Severe traumatic stress may chronically disrupt pair bonding, whereas mild acute stress facilitates it. Although most of the reports using a rat SPS model focus on mechanisms underlying anxiety-like behaviors, only one report indicates that the SPS treatment impairs social recognition of rat [44]. It is still unknown whether the failure of pair bonding in our study is attributed to impaired social recognition, although the ability of social recognition is indispensable for the expression of partner preference. SPS-treated voles significantly preferred to stay in the chamber containing a stranger female (Fig. 3B), suggesting the ability to discriminate between partner and stranger females. This is consistent with previous reports showing that inhibition of partner preference by the pharmacological blockade of V1aR antagonist or μ -opioid receptor accompanies increased duration spent in the chamber

containing a stranger female, compared with that pair bonded male voles do [35,45]. It will be necessary to clarify the effect of traumatic stress on social recognition in future studies.

Interestingly, oral administration of paroxetine prevented SPS-mediated impairments of pair bonding at a clinically relevant dose (Fig. 4). Oral administration of paroxetine alleviates enhanced fear response in SPS-treated rats [15] and reduces cue-induced freezing behavior in SPS-treated mice [46]. The FDA approves paroxetine to be prescribed for PTSD patients. Our results indicate that SSRI including paroxetine may alleviate the disturbances of pair bonding in PTSD patients, although this should be tested empirically. However, we should not exclude a possibility that paroxetine primarily facilitates pair bonding, since we did not administer this drug to control voles. There are a few reports indicating the significance of a serotonin system on social behavior in prairie voles. Chronic administration of fluoxetine, another SSRI, suppresses aggressive behavior in male prairie voles [47]. Perinatal exposure to 5-methoxytryptamine (5-MT), a non-selective serotonin agonist, decreases oxytocin and vasopressin IRs at the PVN, as well as serotonin IR at the amygdala [48]. 5-MT treated male voles showed decreased affiliation and increased anxiety-like behavior. In mice, intracerebroventricular administration of oxytocin stimulates serotonin release, which decreases anxiety-like behavior through the activation 5-HT_{2A/2C} receptors [49]. Further, a recent report indicates that the rewarding properties of social interaction in mice require the coordinated activity of oxytocin and serotonin in the NAcc [50]. Oxytocin release in the NAcc from PVN neurons causes the release of serotonin from terminals of serotonergic neurons from the dorsal raphe. Activation of 5-HT_{1B} receptors on glutamatergic terminals from various brain regions decrease presynaptic function at excitatory synapses onto medium spiny neurons in the NAcc, which is implicated in social preference [50]. Together with these reports, our results imply the involvement of a serotonin system in the formation of pair bond in prairie voles. This idea is consistent with a very recent report indicating that a subset of inhibitory serotonergic neurons projecting to the anterior hypothalamus mediate spatiotemporal release of neuropeptides in modulating affiliation [51].

Although SPS treatment induces enhanced anxiety-behavior and anhedonia [52,53], we did not detect such abnormality in our model (Fig. 2). These differences appear to result from the differences of experimental conditions, rather than species differences. Enhanced anxiety behavior is revealed by an elevated plus maze test, whereas we performed an open field test. In a mouse SPS model, SPS-treated mice exhibit more cue-induced fear behavior in response to a tone given during a SPS treatment, but not in the absence of tone [46]. Further, housing condition, in which subjects including sham-treated voles were individually housed during the experimental schedule, may make it difficult to detect enhanced anxiety behavior. Social isolation in adult male mice reduces the duration spent in a center area of an open field test [54]. We should not exclude the possibility that SPS treatment affects social and emotional behaviors from the results we show here, since our preliminary data shows that SPS treatment enhances fear conditioning in prairie voles (unpublished data). SPS-induced anhedonia is revealed by the comparison of sucrose uptake under varied concentration of sucrose. At 1% of sucrose which we adopted, there is little difference of sucrose uptake between SPS-treated and control rats, whereas the difference is greater at 0.25% of sucrose [53].

There were no differences in the expression levels of GR and MR between control and SPS-treated voles in the experiment 3 (Table 2). SPS treatment affects the expression of GR and MR, although the effects are varied among reports. Liberzon et al. [12] reports that GR mRNA is up-regulated while MR mRNA is down-regulated at the rat hippocampus 7 days after SPS treatment. Another report indicates that IRs of both GR and MR are reduced at the hippocampus in SPS-treated rats [55]. In contrast, in a mouse SPS model, GR mRNA is increased 7 days after SPS treatment, while no difference is observed in GR IR between control and SPS-treated mice [46]. Further analyses are necessary to verify SPS-treated voles as a PTSD model. SPS-treated voles showed the reduced IR of oxytocin in the SON, but not in the PVN 7 days after SPS treatment (Fig. 5, Table 2). Oxytocin neurons at the SON may be more vulnerable to the disturbance of the serotonin system than those at the PVN. However, oxytocin IR at the SON in SPS-treated voles was comparable to that in untreated voles 14 days after SPS treatment (Table 1). It should be noted that the temporal aspects of the reduction of oxytocin IR was inferred from two independent experiments, experiment 1 and 2. Interestingly, oxytocin and serotonin reciprocally regulate the secretion of each neurotransmitter. An intraperitoneal injection of oxytocin at postnatal day 1 affects innervation of serotonin neurons at day 21 [56]. Conversely, serotonin stimulates synthesis and secretion of oxytocin and vasopressin [57]. The disturbance of oxytocin and/or serotonin before cohabitation may interfere with the formation of pair bond. SPS treatment transiently enhances vasopressin IR in the SON in rats, but not in the PVN, 5–6 days after the treatment [58]. Such enhancement returns to basal levels within 7 days. SON neurons may be more sensitive to SPS treatment than PVN neurons, although we did not observe significant change of vasopressin IR in these nuclei.

When a partner vole was presented to subject voles in experiment 2, SPS treatment significantly increased number of cells double-labeled with oxytocin and cFos (Table 1). Presentation of a partner female induces cFos IR at various brain regions including PVN [59]. However, it is unlikely that enhanced response of oxytocin neurons at the SON contributes to the disturbance of pair bonding, since paroxetine administration did not prevent the enhancement. We need to investigate neuronal circuits previously identified as a pair bonding network (oxytocin, social information processing and reward circuitry) and the serotonergic system which has been shown to modulate social reward but has not been investigated in relation to pair bonding [50], in order to identify the circuit disturbed by the SPS treatment.

Here, we showed that traumatic events such as a SPS paradigm disturb the formation and/or expression of a pair bond. The amelioration of the SPS-induced impairment of the pair bond by oral administration of paroxetine suggests the involvement of the serotonergic system in pair bonding. Immunohistochemical analyses suggested the SPS treatment affects oxytocin neurons at the SON, although the effect appears to be unrelated to the disturbance of pair bonding by SPS treatment. Our model may be useful to understand the neuronal mechanism underlying social problems caused by traumatic events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

GR	glucocorticoid receptor
IR	immunoreactivity immunoreactive
LS	lateral septum
MR	mineralocorticoid receptor
MeA	medial amygdala
NAcc	accumbens nucleus
pBNST	principal bed nucleus of the stria terminalis
PBS	phosphate buffered saline
PBS-T	PBS containing 0.3% Triton X-100
PTSD	post-traumatic stress disorder
PVN	paraventricular nucleus
SON	supraoptic nucleus
SPS	single prolonged stress
SSRI	selective serotonin reuptake inhibitor
TH	tyrosine hydroxylase
V1aR	vasopressin 1a receptor
VP	ventral pallidum

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbr.2016.08.022>.

Highlights

- Single prolonged stress (SPS) disturbed the formation of pair bond in prairie voles.
- The administration of SSRI prevented the disturbance of pair bonding.
- SPS transiently decreased oxytocin levels in the SON.
- SPS affected the response of oxytocin neurons in the SON to exposure to a partner.
- SPS-treated voles may be suitable to study the effect of trauma on social bonding.

Fig. 1.

Experimental schedules. (A) Experiment 1 was designed to clarify the effects of SPS on behaviors. Subjects were individually housed 4 days before the SPS treatment (at Day 0). They were tested for sucrose preference at Day -1 and Day 7. Subjects were cohabitated with a female for 6 days (box with oblique lines, Day 8–14) and investigated for partner preference at Day 14. (B) Experiment 2 was designed to clarify whether paroxetine prevented the disturbance of pair bonding by the SPS treatment. Subjects were orally administrated with paroxetine between Day 1 and 10 (gray box), and cohabitated with a female vole for 3 days (box with oblique lines, Day 7–10). At Day 11, subjects were tested for partner preference and individually housed for 3 days. Brains were prepared at Day 14, 90 min after subjects were presented to their partner female. (C) Experiment 3 was performed to prepare brains from SPS- and sham- treated voles without stimulations 7 days after SPS treatment. a, Three of sham-treated voles and one SPS-treated vole were eliminated due to a technical problem. A sham-treated vole showing no sucrose preference was also eliminated from the analysis.; b, Three voles were died during the SPS treatment.; c, Totally 7 voles were eliminated because of technical trouble and death.; d, Two of sham-treated voles showing sexual behavior to stimulus voles were eliminated from the analysis.; e, One vole in each treatment was eliminated because of a technical problem.; f, Brain of sham-treated vole administrated with H₂O was eliminated because of inadequate perfusion.

Fig. 2.

SPS treatment did not induce either anhedonia or anxiety-like behavior. (A) Anhedonia test. Both sham- and SPS- treated voles preferred to drink water containing 1% sucrose. $n = 8$ for sham treatment, $n = 11$ for SPS treatment. (B) Open field test. SPS-treated voles spent time in the center area as much as sham-treated voles did. $n = 11$ for sham treatment, $n = 10$ for SPS treatment.

Fig. 3.

SPS treatment disturbed pair bonding. (A) Duration of huddling behavior. SPS-treated voles huddled with their partner female as much as with a stranger female, whereas sham-treated voles significantly spent more time to huddle with their partner female than a stranger female. (B) Duration to spent in a chamber containing a partner or stranger female. SPS-treated voles preferred to stay in the chamber containing a stranger female than one containing partner female. Contrarily, sham-treated voles spent more time in the chamber containing their partner than one with a stranger female. **, $P < 0.01$. (C) The numbers of entries into chambers. There was no difference among conditions. $n = 9$ for sham treatment, $n = 9$ for SPS treatment.

Fig. 4.

Prevention of SPS-mediated impairment in pair bonding by paroxetine administration. (A) Duration of huddling behavior. When paroxetine was orally administered, SPS-treated voles significantly spent more time to huddle with their partner female than a stranger female. When only distilled water was administered, SPS-treated voles did not show the partner preference. (B) Duration to spent in a chamber containing a partner or stranger female. SPS-treated voles indiscriminately spent time in a chamber containing a partner or stranger female, regardless of whether they were administered with paroxetine. **, $P < 0.01$. $n = 11$ for SPS treatment and administered with paroxetine, $n = 11$ for SPS treatment and administered with the vehicle.

Fig. 5.

Immunoreactivities of oxytocin and cFos at the PVN (A, C, E) and SON (B, D, F). Oxytocin IR (bluish gray) was observed at somatic bodies, while cFos IR (brown) was in nuclei. The surrounded area is magnified at the right bottom in (A). An arrow indicates a cell double-labeled with oxytocin and cFos IRs. The number of such cells was counted. Open arrowheads show cells labeled with oxytocin IR but not cFos. (A, B) SPS-treated voles administrated with only vehicle. (C, D) SPS-treated voles administrated with paroxetine. (E, F) Voles without any treatment nor administration. v, 3rd ventricle; ot, optic tract. Bars, 100 μ m..

Fig. 6. Immunoreactivities of oxytocin (A–D) and vasopressin (E–H). Immunoreactivities were observed at somatic bodies and neurites in the PVN (A, B, E, F) and SON (C, D, G, H). Localization of oxytocin and vasopressin was similar between sham- (A, C, E, G) and SPS- (B, D, F, H) treated voles. Oxytocin IR at the SON appeared to be higher in sham-treated voles than SPS-treated voles (C, D). Bars, 100 μ m. v, 3rd ventricle; ot, optic tract.

Table 1

Numbers of cells double-labeled with cFos as well as oxytocin or vasopressin.

Antigen/Area	Untreated	SPS + H2O	SPS + PRX	F	P
Oxytocin					
SON	5.94 ± 0.34	5.28 ± 0.42	5.42 ± 0.34	$F_{2,28} = 0.434$	$P = 0.652$
Oxytocin/cFos					
PVN	2.00 ± 0.42	2.42 ± 0.47	2.42 ± 0.51	$F_{2,29} = 0.205$	$P = 0.816$
SON	0.29 ± 0.18	1.70 ± 0.26 ^{**}	1.36 ± 0.34 [*]	$F_{2,25} = 5.38$	$P = 0.011$
Vasopressin/cFos					
PVN ^a	3.00 ± 0.80	1.10 ± 0.28	1.55 ± 0.34	$F_{2,14,4} = 2.55$	$P = 0.113$
SON	3.13 ± 0.52	3.40 ± 0.31	2.58 ± 0.60	$F_{2,27} = 0.732$	$P = 0.490$

* $P = 0.05$ vs Untreated.

** $P = 0.01$ vs Untreated.

^a Welch test was performed, since sample variances were unequal.

Table 2

tensities of immunoreactivities for GR, MR, vasopressin, oxytocin, and TH.

Antigen/Area	Sham	SPS	T	P
GR				
Hippocampus	3.91 ± 0.36 (n = 3.0 ± 0.0)	3.54 ± 0.098 (n = 3.0 ± 0.0)	T ₁₂ = 0.851	P = 0.357
MR				
Hippocampus	3.12 ± 0.24 (n = 3.0 ± 0.0)	2.66 ± 0.14 (n = 3.0 ± 0.0)	T ₁₂ = 1.537	P = 0.150
Vasopressin				
PVN	27.78 ± 6.12 (n = 4.0 ± 0.3)	23.81 ± 4.00 (n = 3.8 ± 0.3)	T ₁₄ = 0.543	P = 0.596
SON	6.54 ± 0.49 (n = 4.6 ± 0.4)	6.94 ± 0.84 (n = 4.3 ± 0.6)	T ₁₄ = 0.407	P = 0.690
Oxytocin				
PVN	11.53 ± 1.78 (n = 5.0 ± 0.5)	11.18 ± 1.18 (n = 4.8 ± 0.3)	T ₁₄ = 0.164	P = 0.872
SON	7.75 ± 1.06 (n = 5.0 ± 0.4)	4.85 ± 0.68 (n = 3.6 ± 0.4)	T ₁₄ = 2.305	P = 0.037*
TH				
MeA	2.59 ± 0.34 (n = 3.2 ± 0.4)	2.25 ± 0.45 (n = 2.6 ± 0.4)	T ₁₄ = 0.603	P = 0.556
PVN	3.15 ± 1.05 (n = 4.0 ± 0.3)	2.47 ± 0.55 (n = 4.1 ± 0.3)	T ₁₃ = 0.547	P = 0.594
pBNST	3.82 ± 0.31 (n = 1.1 ± 0.1)	3.26 ± 0.36 (n = 1.2 ± 0.1)	T ₁₄ = 1.192	P = 0.253
LS	2.30 ± 0.44 (n = 6.5 ± 0.5)	1.88 ± 0.19 (n = 7.9 ± 0.4)	T ₁₄ = 0.867	P = 0.401