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cis-3-Hexenol and *trans*-2-hexenal mixture prevents development of PTSD-like phenotype in rats

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HIGHLIGHTS

- Daily, not acute, green odor (GO) presentation facilitated fear extinction.
- Daily GO presentation prevented development of PTSD-like phenotype after fear conditioning.
- Chronic paroxetine treatment improved PTSD-like phenotype but not fear expression.
- Chronic *p*-chlorophenylalanine (PCPA) treatment inhibited fear extinction but not PTSD-like phenotype.
- The alleviative effects of GO were masked by paroxetine and abolished by PCPA.

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ABSTRACT

Several green leaf volatiles have anxiolytic/antidepressant properties and attenuate adrenocortical stress response in rodents. However, it remains unknown whether a mixture of *cis*-3-hexenol and *trans*-2-hexenal so-called 'green odor (GO)' affects fear-associated post-traumatic stress disorder (PTSD)-like behavior. In the present study, fear memory of the initial conditioning stimulus was stably maintained by weekly presentation of conditioned tone. Examination of open field behavior, acoustic startle response, prepulse inhibition, and immobility in the forced swim test for 2 weeks after initial conditioning revealed that conditioned rats sustained anxiety, enhanced startle response, hypervigilance, depression-like behavior, and hypocortisolism, which is consistent with PTSD symptoms. Daily, not acute, GO presentation facilitated fear extinction and reduced PTSD-like behavioral and endocrinal responses. To further investigate the mechanism of effect of GO, we examined the effect of paroxetine (a selective serotonin reuptake inhibitor), *p*-chlorophenylalanine (PCPA, an irreversible serotonin synthesis inhibitor), alone or in combination of GO on PTSD-like phenotype. The alleviative effects of GO were masked by simultaneous paroxetine administration. PCPA-induced serotonin depletion abolished the effects of GO. Our results suggest that daily GO presentation facilitates fear extinction and prevents development of PTSD-like symptoms.

1. Introduction

Psychological stress is a precipitating factor in psychiatric disorders such as post-traumatic stress disorder (PTSD) and depression. Some humans and animals that have experienced a severely stressful situation exhibit traumatic memory formation and behavioral alterations, such as long-lasting fear responses to reminders of the stressful event, enhanced startle response, hypervigilance, anxiety, a depressive state, and abnormal activation of the hypothalamic-pituitary-adrenal (HPA) axis [1–5].

Green odor (GO), a mixture of *cis*-3-hexenol and *trans*-2-hexenal, attenuates fear, anxiety and stress responses in rodents [5–8]. In humans, GO affects pleasantness and alters the P-300

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component of the event-related potential [9]. Our previous studies have demonstrated that GO reduces hyperthermia and attenuates plasma adrenocorticotropic hormone elevation in rats exposed to psychological stressors such as immobilization, predator odor, and footshock [7,8,10]. Other studies have demonstrated that GO also has anxiolytic and antidepressant effects in rodents in the forced swim and two-way conditioned avoidance tests [5,11]. These studies suggest that GO presentation attenuates psychological stress-induced behavioral and endocrine responses. However, it remains unknown whether GO affects PTSD-like phenotype following initial fear conditioning.

In the present study, we subjected rats to fear conditioning and a series of subsequent tests examining open field behavior, acoustic startle response, prepulse inhibition (PPI), and forced swim behavior. Conditioned rats exhibited a long-lasting fear, anxiety-, depression-like behavior, and attenuated HPA axis activity, which is consistent with PTSD symptoms [1–4]. We then examined whether GO would alleviate this PTSD-like phenotype, and compared its effects with those of the selective serotonin reuptake inhibitor (SSRI) paroxetine, irreversible serotonin synthesis inhibitor *p*-chlorophenylalanine (PCPA), alone or in combination with GO.

2. Materials and methods

2.1. Animals

Experiments were carried out in male Sprague-Dawley rats (7 weeks old). Animals were individually housed in plastic cages ($14 \times 21 \times 12$ cm) containing wood chips and covered with a wire lid. The cages were kept in a room maintained at $24 \pm 1\,^{\circ}$C, under a 12-h light/dark cycle (lights on at 09:00) for 7 days before the start of the experiment. Animals could access food and water ad libitum. To minimize responses to experimental manipulation, all rats underwent daily 10 min habituation sessions until the start of the experiment, and were allowed to acclimate to the experimental room for 30 min each day before any experimental procedures. All procedures were performed between 11:00 and 16:00. All experiments were conducted in accordance with the Guidelines for Animal Research of the Physiological Society of Japan and the Hirosaki University of Medicine.

2.2. Drugs

Paroxetine hydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was diluted to 10 mg/ml with distilled water. *p*-Chlorophenylalanine methyl ester (PCPA, Sigma, St. Louis, MO) was dissolved in 0.9% saline (300 mg/ml). Twenty-four hours after fear conditioning, paroxetine (1.0 ml/kg body weight) was injected intraperitoneally, at 11:00, or 30 min before the behavioral tests, daily for 2 weeks (Fig. 1a) [12]. For serotonin depletion, rats received intraperitoneal injection of PCPA (1.0 ml/kg body weight) at 11:00 on Day 2, 3 and 11. This chronic PCPA administration schedule decreased rat brain serotonin levels by about 83.5% [13].

2.3. Odor solution and presentation

GO comprised equal amounts of *cis*-3-hexenol (Wako Pure Chemical Industries) and *trans*-2-hexenal (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) diluted with distilled water to 0.03% (v/v) [8,9,11]. Distilled water was used as a control odor. For acute GO presentation experiment, rats were exposed to 200 μl odor solution-impregnated cotton in their home cage for 60 min (11:00–12:00) 24 h after fear conditioning [10]. For daily odor presentation experiment, animals were exposed to odor in their home cage for 60 min (11:00–12:00), daily except on behavioral test days (Fig. 1a). In the open field and PPI tests, four pieces of cotton were impregnated with odor (50 μl/cotton piece) and placed in each corner of the test apparatus 30 min before the start of each test. In the retention test, odor was delivered from a port in an odor exposure chamber ($32 \times 26 \times 21$ cm) by a custom-made olfactometer (Bio Research Center, Co., Ltd., Tokyo, Japan) at a constant flow of 1.0 L/min [14,15]. In the forced swim test, the odor was delivered by an olfactometer 10 cm above the center of the water surface. Rats treated with paroxetine or PCPA in combination with GO were exposed to GO for 60 min immediately after each drug administration in their home cage or during each behavioral test 30 min after injections.

2.4. Experimental protocol

We designed a new experimental protocol based on previous studies to investigate the development of PTSD-like behavior after fear conditioning (Fig. 1a) [8,16–18]. Rats were randomly assigned to four groups ($n = 6$ per group): no fear conditioning (NF); fear conditioning (FC); acute or daily treatment with GO after fear conditioning (FC + aGO; FC + dGO); and fear conditioning followed by chronic administration of paroxetine (FC + PR), PCPA (FC + PC), alone or in combination with GO (FC + PR + dGO; FC + PC + dGO).

2.4.1. Fear conditioning and retention test

Fear conditioning was conducted in a Plexiglas footshock chamber ($32 \times 26 \times 21$ cm) containing a metal grid floor that could deliver electric shocks (FreezeScan, CleverSys Inc., Reston, VA, USA), as previously described, with modifications [8,16,17]. Twenty-four hours after a chamber habituation session (10 min, no tones or shocks), rats underwent fear conditioning, in which a pure tone (105 dB, 3 kHz, 10 s; CS) was immediately followed by a footshock (0.5 mA, 5.0 s; unconditioned stimulus, US). Ten CS/US stimuli were presented at 60–120 s intervals. Animals in the NF group were subjected to 10 presentations of the tone alone. To investigate fear memory retention, the retention test (10 presentations of the CS alone) was performed in the odor exposure chamber. Fear-induced freezing (cessation of all movement except for breathing) was measured for 60 s per trial as follows: % Freezing = [duration of freezing behavior (s)/60 s] × 100. Freezing was calculated in blocks of two trials during fear conditioning and the retention tests [17].

2.4.2. Acoustic startle and PPI tests

Hamilton Kinder PPI equipment (SM-100, Kinder Scientific, Poway, CA, USA) was used to measure acoustic startle response, sensorimotor gating and attention. Acoustic startle and PPI protocols used were previously described [18]. The amount of PPI was calculated as: % PPI = [1−{startle amplitude in prepulse–pulse trial (N)}/{startle amplitude in pulse alone trial (N)}] × 100

2.4.3. Open field test

The behavior of rats in an open field ($60 \times 60 \times 60$ cm, 50 lx) was recorded for 10 min using a video tracking system (CaptureStar, CleverSys Inc.). Horizontal locomotor activity [total distance moved (m)] and percent time spent in the center 25% of the arena were analyzed by TopScan behavioral analysis software (CleverSys Inc.). Animals were placed in different open field arenas across tests to avoid spatial learning.

2.4.4. Forced swim test

Rats were introduced to a Plexiglas cylinder (diameter 25 cm, height 40 cm) filled with warm water ($25 \pm 1\,^{\circ}$C, height 18 cm) for 5 min to examine depression-like state. Behavioral responses were recorded using a video tracking system (ForcedSwimScan, CleverSys Inc.). Time spent immobile (absence of directed head or body