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Effects of Green Odor on Expression of Fos-immunoreactivity in the Paraventricular Nucleus of the Thalamus in Forced Swimming Rats

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Introduction

The paraventricular nucleus of the thalamus (PVT) projects to the limbic forebrains such as the prefrontal cortex, amygdale and nucleus accumbens (Otake *et al.*, 2002) and is implicated directly or indirectly in the generation of behavioral, endocrine and/or autonomic responses to stress (Spencer *et al.*, 2004). These stress-induced responses include activation of hypothalamic–pituitary–adrenal axis and increase of body temperature. Veening *et al.* (2004) have reported that elevations of plasma corticosterone level and body temperature induced by stress are associated with an increase of Fos-immunoreactivity (Fos-IR) in the PVT. Fos-IR in the PVT also increases under a wide variety of stresses such as forced swimming and restraint (Otake *et al.*, 2002). Recently, it has been demonstrated that the so-called 'green odor' emanating from green leaves attenuates stress-induced elevations in plasma ACTH level and body temperature in rats (Akutsu *et al.*, 2002; Nakashima *et al.*, 2004), suggesting that green odor may inhibit the increase of Fos-IR in the PVT induced by stress. Thus, we examined the effects of green odor on the expression of Fos-IR in the PVT in forced swimming rats.

Materials and methods

Experiments were performed on male Wistar rats at 8 weeks of age (Sankyo Lab., Shizuoka, Japan) and approved by the Institutional Animal Care and Use Committee of the Faculty of Engineering of Toyama University. Rats were housed in a light-controlled room (light on, 06:00–18:00) at a temperature of 23 ± 1°C. Food and water were available *ad libitum*. Weight-loaded forced swimming was performed as described previously (Tanaka *et al.*, 2003). In each rat, a load of steel ring weighing ~6% of body weight was attached to the proximal end of the tail. Rats were then placed individually in plastic cylinders (height 42 cm, diameter 29 cm) containing water 27 cm deep at 23°C, allowed to swim for 20 min and transferred to their recovery cage covered by a transparent acrylic plate with a small hole (3 × 3 cm) at a corner. In the recovery cage, rats inhaled *trans*-2-hexenal (HEX), a main substance in green odor (swim + HEX group), or triethyl citrate (TEC), a solvent of HEX (swim + TEC group). The concentration of HEX was 0.3% (w/w). To supply the odors to the recovery cage, room air was passed through an air-tight bottle containing 0.3% HEX or TEC with an air pump (700 ml/min) and then led to the recovery cage via a Teflon tubing. Control rats were maintained in the recovery cage without forced swimming and inhalation of odors such as HEX or TEC. After rats spent 60 min (80 min for control rats) in their recovery cage, they were anesthetized with ether, and perfused with 0.9% normal saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS, pH 7.4). Brains were then removed from the skull, and post-fixed in the same fixative solution for 12 h at 4°C and stored in 15% sucrose solution in 0.1 M PBS at 4°C. Frozen sections (50 µm) were cut in frontal planes on a microtome and collected and stored in 0.1 M PBS at 4°C. Free-floating sections were rinsed three times for 5 min in 0.3% Triton X-100 in 0.1 M PBS (PBST) and incubated for 30 min in 0.3% PBST containing 0.3% H_2O_2 at 27°C. After treatment with 5% normal goat serum (Vector Lab., CA) for 2 h, sections were incubated with a polyclonal rabbit anti-Fos antibody (Oncogene, CA, USA) at a dilution of 1:20 000 for 24 h at 27°C. Three washes for 5 min in 0.3% PBST were performed between both steps. Sections were then incubated at 27°C with biotinylated anti-rabbit antibody (Vector Lab., CA) for 1 h, followed by amplification with an avidin–biotin–horseradish peroxidase complex (Vectastain ABC-PO kit) for 1 h. Following the washing steps by 0.3% PBST (once) and 0.05 M Tris buffer (twice), sections were visualized with diaminobenzidine (DAB; Dojindo, Kumamoto, Japan) for 10–15 min. Finally, sections were mounted on glass slides, air dried, dehydrated, cleared and coverslipped. Sections from the three groups were processed simultaneously using the same solution. The section including the PVT at 1.4 mm caudal to bregma (Paxinos and Watson, 1986) was selected in each rat and the number of Fos-IR cells was counted by using a digital camera fitted with a light microscope. Data were expressed as means ± SEMs. For statistical analysis, one-way ANOVA followed by a *post hoc* Fisher's least significant difference (LSD) test was used. $P < 0.05$ was taken as the level of statistical significance.

Results

Fos-IR cells in the PVT in the control ($n = 4$), swim + TEC ($n = 4$), and swim + HEX ($n = 4$) groups are shown in Figure 1A, B and C, respectively. The numbers of Fos-IR cells were 11.8 ± 5.5, 30.3 ± 5.1 and 15.8 ± 1.8 in the control, swim +TEC, and swim + HEX groups, respectively (Figure 2). One-way ANOVA demonstrated a signifi-

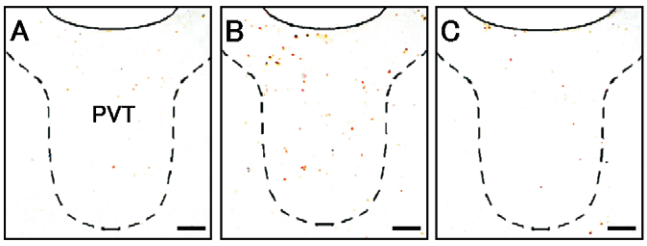

Figure 1 Fos-IR cells in the PVT in the control **(A)**, swim + TEC **(B)** and swim + HEX **(C)** groups. Scale bars = 100 µm.

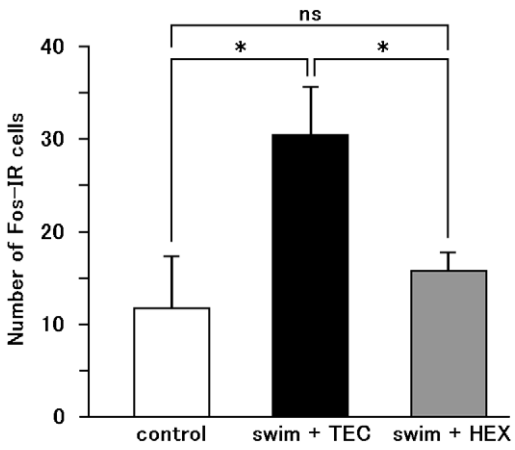

Figure 2 The number of Fos-IR cells in the PVT in the control (left), swim + TEC (middle), and swim + HEX (right) groups. The control and swim + HEX groups showed a significant decrease of Fos-IR cells as compared to the swim + TEC group. There was no significant difference in the number of Fos-IR cells between the control and swim + HEX groups. *$P < 0.05$. ns, no significance.

cant difference in the number of Fos-IR cells between three groups [$F(2,9) = 4.72$, $P < 0.05$]. A *post hoc* Fisher's LSD test further revealed that the number of Fos-IR cells was significantly smaller for the control ($P < 0.05$) and swim + HEX ($P < 0.05$) groups than for the swim + TEC group and there was no significant difference between the control and swim + HEX groups.

Discussion

In our preliminary study, we found that the expression of Fos-IR in the PVT induced by forced swimming was not affected by TEC because the number of Fos-IR cells in the forced swimming alone group was not different significantly from that of the TEC-treated forced swimming group. Therefore, the present results indicate that green odor reduces significantly the expression of Fos-IR in the PVT normally activated by forced swimming. According to anatomical studies, Spencer *et al.* (2004) suggested that the PVT could play a pivotal role in modulating forebrain processing of stress-related information. In addition, recent studies show that stress-induced elevations in plasma ACTH level and body temperature are attenuated by green odor (Akutsu *et al.*, 2002; Nakashima *et al.*, 2004). Taking these findings into consideration, the present results suggest a possibility that green odor has relieving effects on the stress-induced responses through its inhibitory action to PVT neurons.

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