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Research report

# Different patterns of neuronal activities in the infralimbic and prelimbic cortices and behavioral expression in response to two affective odors, 2,5-dihydro-2,4,5-trimethylthiazoline and a mixture of *cis*-3-hexenol and *trans*-2-hexenal, in the freely moving rat

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ABSTRACT

The medial prefrontal cortex (mPFC) is involved in stimulus perception, attentional control, emotional behavior, and the stress response. These functions are thought to be mediated by the infralimbic (IL) and prelimbic (PL) subregions of mPFC; however, few studies have examined the roles of IL and PL cortices in olfactory cognition. In the present study, we investigated the acute effects of two odors, 2,5-dihydro-2,4,5-trimethylthiazoline (TMT) and a mixture of *cis*-3-hexenol and *trans*-2-hexenal (green odor: GO), on behavioral responses and IL and PL neuronal activities using extracellular single-unit recordings in a freely moving rat. We found that the total number of spike firings in IL and PL neurons did not change with 10 s presentation of odors. TMT presentation induced significant changes in burst firing activity in IL and PL neurons, while GO presentation induced changes in burst firing only in IL neurons. In the temporal profile of the firing activity of IL neurons, TMT exposure induced transient activation and GO exposure induced sustained activation. Those of PL neurons showed sustained activation during TMT exposure and transient activations during GO exposure. GO exposure induced a stretch-attend posture, whereas TMT exposure induced immobility. Furthermore, multiple regression analysis indicated that the property of the odor and neuronal activities of IL and PL regions were correlated with behavioral responses. These findings reveal that olfaction-related neurons exist in IL and PL regions, and that the neurons in these regions might temporarily encode odor information in order to modulate motor outputs by tuning firing properties in the early stage of cognition according to the odor property.



## 1. Introduction

The prefrontal cortex (PFC) in primates and non-primates is thought to integrate information and play an important role in a wide variety of tasks, such as working memory formation, top-down control of attention, decision-making, affective processing, behavioral response strategies, and stress responses [2,10,16,19,37]. Although many studies have examined the role of PFC in visual attention tasks in macaque monkeys and human subjects [19] and learning tasks in rats [16], few studies have investigated the role of PFC in olfactory perception tasks [2,8,29,39]. One study showed that the medial PFC (mPFC) mediates the behavioral response to odor cues rather than olfactory recognition memory in ewes [2]. Some studies showed that olfactory stimulation activates *c-fos* expression in this region in rodents [4,9,33], and the orbitofrontal cortex plays an important role in encoding olfactory information to modulate behavioral responses to reward and decision-making correlating with pleasantness and unpleasantness [8,29]. mPFC is functionally dissociable in infralimbic (IL) and prelimbic (PL) regions. The IL region is thought to mediate the initiation of psychogenic stress-induced stress responses, while the PL region might serve to inhibit them [37]. A recent study demonstrated that IL and PL subregions of mPFC play different roles in the expression and extinction of auditory fear conditioning [31]. This indicates that mPFC processes various sensory inputs and outputs; however, the question of whether odor stimulation influences these subregions of mPFC remains, and if so, how do mPFC neurons encode olfactory stimuli? We hypothesized that the IL and PL regions process olfactory inputs and outputs to other brain regions, such as the visual system [19], and focused on pyramidal neurons in cortical layer 5, thought to be driven by inputs from the sensory cortex, limbic area, midbrain, and brainstem [11,26], and output to other cortical and subcortical regions [7,38]. Pyramidal neurons show spike firing and burst firing, assuming that these neuronal activities transmit

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qualitatively different information between neurons [3]; therefore, we investigated these firing properties of IL and PL neurons in olfactory perception using extracellular recordings.

The present study was performed with two affective odors: 2,5-dihydro-2,4,5-trimethylthiazoline (TMT) and a mixture of *cis*-3-hexenol and *trans*-2-hexenal (green odor: GO). Olfactory stimulation of TMT, a component of fox urine odor, is known to induce innate fear and stress responses in rodents. TMT induces *c-fos* activation of various brain regions, such as the amygdala, orbitofrontal cortex, and ventral tegmental area (VTA) [4,27,33], and increases dopamine turnover rates in the dorsal and ventral mPFC and amygdala in SD rats [20]. Also, TMT activates *c-fos* expression in mPFC, the nucleus accumbens, and VTA in mice [9]. Our previous studies showed that TMT exposure induces a freezing response and the hypothalamic-pituitary-adrenal (HPA) axis stress response in Wistar rats in a novel environment [23] and excites adrenal sympathetic nerve activity (ASNA) of the sympathetic-adrenal-medullary (SAM) axis via the histaminergic H1 receptor in anesthetized Wistar rats [12]. These findings indicate that at least TMT activates several cortical and subcortical regions and affects the emotional behavior of rodents as a negative (fear or aversive) odor, although the effect of TMT is thought to depend on the strain and species [6,17,32].

GO, consisting of two plant-derived odorants (e.g. *cis*-3-hexenol and *trans*-2-hexenal), is known to attenuate the stress responses and to have an anxiolytic effect. GO attenuated restraint stress-induced *c-fos* activation in the hypothalamic paraventricular nucleus, paraventricular thalamic nucleus and amygdala in Wistar rats [13], and the anxiolytic effect of GO was mediated by the brain 5-HT system, including the frontal cortex in mice [22,35]. Also, GO attenuated the stress-induced HPA axis response and hyperthermia [1,21]; however, GO tended to weakly influence the HPA axis and behavioral responses or not in both familiar and unfamiliar environments [23], suggesting that the effect of GO might depend on the presence of stressors or the emotional state during presentation. In humans, GO presentation induces pleasantness and decreases in event-related potential P-300, reflecting cognitive processes [30]; therefore, for mammals, information on GO ascends and descends to the brain regions involved in attenuation of the stress response and anxiolysis, and exerts positive (pleasant or calming) effects on emotional behavior and the stress response.

Overall, we expect that these affective odors (positive or negative) provide useful clues for evaluating the effect of olfaction on mPFC. Behavioral responses to these odors also provide important suggestions about cognitive functions; therefore, we investigated neuronal activities in IL and PL subregions of mPFC, behavioral response and the correlation between them in response to acute presentations of TMT and GO using extracellular recordings in freely moving rats.

## 2. Materials and methods

### 2.1. Animals

The experiments were carried out on male Wistar rats (8 weeks old, $n = 7$) purchased from Shimizu Laboratory Supply (Kyoto, Japan). They were individually housed in plastic cages ($30 \times 35 \times 18$ cm), called a home cage, with wood chips, and covered with wire lids in the animal room maintained at $24 \pm 1$ °C with a 12-h light/12-h dark cycle (lights on at 6:00). Animals could access food and water *ad libitum*. All procedures were performed between 11:00 and 14:00. All experiments were conducted in accordance with the Guidelines for Animal Research of the Physiological Society of Japan.

### 2.2. Odor solutions

2,5-Dihydro-2,4,5-trimethylthiazoline: TMT (Contech, Delta, British Columbia, Canada) is known as a predator-related odor. In this study, animals were exposed to 9.67 μl TMT, an amount that provoked increases in plasma ACTH and corticosterone concentrations, adrenal sympathetic nerve activity and the freezing response [4,12,23]. A mixture of equal amounts of *trans*-2-hexenal and *cis*-3-hexenol (Wako Pure Chemicals, Osaka, Japan) diluted with equal amounts of ethanol followed by dilution with distilled water to 0.03% (v/v) was used as the green odor (GO) at 200 μl, which attenuated stress-induced elevations in plasma ACTH concentrations and body temperature [1,21]. Distilled water of 52.8 μl was used as a control, an amount that failed to increase plasma ACTH concentrations and adrenal sympathetic nerve activity [4,12,23]. After cotton had been impregnated with the odor solution ($2 \times 2$ cm) at the start of odor exposure, it was carefully placed in the home cage.

### 2.3. Electrophysiological recording

Chronically implanted stereotrodes were used for single-unit recordings. The original stereotrode (two 50 μm diameter Teflon®-insulated tungsten wires; A-M systems, Inc., Washington, USA) was developed according to previous studies [5,18] and Current Protocols in Neuroscience Unit 6.16 (John Wiley & Sons, Inc., 2002). The stereotrode was implanted under pentobarbital anesthesia (100 mg/kg) in IL and PL subregions of rat medial PFC (AP, 2.9 mm; ML, 0.8 mm; DV, 4.0–5.2 mm; Fig. 1A), according to the atlas of the rat brain [24]. Reference electrodes were placed on the posterior cortical surface to the recording electrodes. Ground wires were connected to stainless steel skull screws. The recording electrode was secured to the cranium with dental cement using anchor scull screws. Animals were allowed 5 days to recover from surgery before the start of experiments. All recording sessions were performed in the home cage, placed inside a Faraday cage. Animals were connected to an FET headstage by means of lightweight cabling that passed through a slipring (Muromachi Kikai, Co., Ltd., Tokyo, Japan) to allow them to move freely. Extracellular unit activity was recorded using modified DPA-100D amplifiers (DIA Medical Systems, Co., Ltd., Tokyo, Japan). The amplified signal was digitized (50 kHz sampling rate), band-pass filtered between 300 Hz to 8000 Hz and saved as an ASCII file using DasyLab ver. 6. (Datalog GmbH, Mönchengladbach, Germany) on a PC. The acquired data were processed by DataView ver. 6.3.2. (developed by Heitler WJ, 2009). Off-line spike sorting was performed with the Spike Sorter Wizard program of DataView, the automatic clustering program KlustaWin ver. 3.1. (developed by Heitler WJ, 2002) and the manual sorting method. KlustaWin implemented unsupervised clustering according to the first 3 principal components of all waveforms recorded from each electrode in 3-dimensional space and performed the initial separation of waveforms into individual units. Each cluster was then checked manually to ensure that the cluster boundaries were well separated and waveform shapes were consistent with spikes. The waveforms in each selected cluster were averaged to produce computer-generated templates that were then used to match spikes recorded during each single session. Obvious artifacts were removed and the stability of clusters throughout the experiment was confirmed by plotting the first principal component versus the timestamp for each waveform. An absolute refractory period of over 1.1 ms was used to select single units. Typically, 1 or 2 neurons were isolated from each electrode. Although recording with chronically implanted electrodes can lead to stable recordings across sessions, spike waveforms and firing properties may change from session to session. To detect the same single unit throughout the experiment, we used the waveform templates defined in the first recording session and the unsupervised clustering method (Fig. 1B).

Based on previously reported criteria [14,25,34], neurons were divided into regular spiking (firing rate <10 Hz, sporadic firing pattern, putative pyramidal neurons) and fast spiking (firing rate >10 Hz, tonic firing pattern, putative interneurons) units; however, because very few units met the criteria of fast-spiking neurons, reliable statistical analysis could not be performed for this subgroup. Thus, all reported results are based on regular-spiking single units.

### 2.4. Procedure

After the surgical recovery period, rats received a daily 30 min habituation session until the experiment days (Day 5–Day 1: Fig. 1C). During habituation sessions, animals were handled for 5 min to minimize responses to the experimental operator and were acclimated to the recording environment for 15 min. During acclimation, non-odor-applied cotton was presented to animals to habituate them to experimental manipulation.

After habituation, to compare acute odor-induced changes in neuronal activity in mPFC, rats received 3–5 odor exposure sessions with ≥120 s intersession intervals (Day 0–Day 2: Fig. 1C). Five minutes after 10 min habituation and 20 s baseline recording session, odor exposure sessions consisting of 10 s pre-odor and odor exposure periods were implemented. Odor-applied cotton was randomly placed in one of the four corners of the home cage during a 10 s odor exposure session. Animals received the odor of distilled water on Day 0, and were randomly exposed to GO and TMT on Day 1 and Day 2.

### 2.5. Data analysis

The electrophysiological data were further analyzed to assess unit characteristics. Mean firing rates between 10 s pre-odor and odor exposure periods and firing rate histograms with 500 ms bins were analyzed. Neurons were considered to be odor-responsive and were included in the experiment if their mean firing rates in odor exposure periods changed by at least an increase of 150% or a decrease of 50%