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Effects of Plant-derived Odors on Sleep–Wakefulness and Circadian Rhythmicity in Rats

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Introduction

Plant-derived odorants promote good feeling, refresh spirits and sometimes relieve various stresses in humans. Physiological and psychological effects of plant-derived volatile chemicals have long been acknowledged in folk medicine and aromatherapy. Recent evidence from animal experiments suggests that these plant-derived chemicals affect various animal behaviors via modulating neural or humoral mechanisms (Torii *et al.*, 1988; Sano *et al.*, 1998; Ilmberger *et al.*, 2001; Akutsu *et al.*, 2002; Nakashima *et al.*, 2004). However, the majority of those animal experiments were performed under unusual environmental conditions (various stresses, anesthesia). Therefore, we examined the effects of these odorants on sleep–wakefulness and circadian rhythm in freely moving animals. We used two volatile substances: α-pinene and mixture of green odor (*n*-hexenal and *n*-hexenol).

Materials and methods

Animals

Male Sprague–Dawley (SD) rats (CD-IGS rat, aged 5 months; Charles River Japan) were used for the sleep–wakefulness study and male microphthalmic (MP, kindly supplied by Professor S. Sugita, Utsunomiya University) rats (aged 10–18 months), which completely lack the optical nerve, for the study of aging effects on circadian locomotor rhythms. They were housed at a controlled temperature (24 ± 1.0°C) and illumination (light 05:00–19:00 h) and given food and water *ad libitum*. Over 7 days before the experiments, SD rats were implanted with electrodes for polygraphic recording under pentobarbital sodium anesthesia (35 mg/kg body wt i.p., nembutal; Dainippon Pharmaceutical). After the surgery, SD rats were housed individually. MP rats were also housed individually prior to the experiments.

Green odor (Soda Aromatic, Tokyo, Japan) and α-pinene (Sigma-Aldrich Co.) were diluted with triethyl citrate (TEC) on the day of the experiments. The concentration of odorants in the present study was 0.3 % (w/w), which was 10 times higher than in previous studies (Sano *et al.*, 1998; Akutsu *et al.*, 2002; Nakashima *et al.*, 2004). We were able to apply these odorants to rats without any disturbance of free-moving behaviors, because we used a new odorant delivery system. In this system, 0.03–0.1% odorants did not show any significant effects on sleep–wakefulness and circadian rhythm (data not shown).

Odorant delivery system

The air, ventilated by an air pump in a flow volume of 3 l/min, was deodorized by passage through a charcoal packed in U-shaped glass tube. A gas-washing glass bottle with 60 ml of 0.3% odorant solution or vehicle (TEC), connected with silicon tube from the U-shaped [SANS U] glass tube, was bubbled by an air pump. The other side of the bottle was connected to a three-way plastic connector for the EEG study or a six-way plastic connector for the rhythm study. Each pathway was connected to a silicon tube and opened through a glass funnel over the top of two cylindrical observation cages (ϕ350 × 400 mm height) or five individual plastic covered cages (300 × 350 × 250 mm) with two ventilation holes. In this system, we attempted to avoid the effect of odorant chemicals through pathways other than the olfactory sensing system. We have also confirmed this in other experiments (paper in preparation) in which the olfactory tract was cut to disrupt odorant-induced changes in this system.

Experimental procedure

Long-term polygraphic records (EEG, EMG, for 7 days; 1 mm/s paper speed, total 605 m length) were obtained simultaneously from two SD rats using an EEG recorder (EEG7410; Nihon Koden Ltd). Vehicle exposures started at 18:00 on the third day and odorant exposures from 18:00 on the fifth day to 12:00 on the sixth day and were performed by changing the gas-washing bottle. The polygraph records were divided into three stages by visual inspection: alertness, slow wave sleep (SWS) and paradoxical sleep (PS). Six SD rats were used for these experiments and four rats were selected for statistical comparison. For statistical analysis, two-way ANOVA followed by the Bonferroni post-tests were applied using Prism 4 (Graphpad Software Inc.).

The circadian locomotor rhythm was measured using an area sensor (F5B; Omron, Tokyo) and the data were stored in a personal computer by using the Chronobiology Kit (Stanford Software Systems, CA). Vehicle exposure started at 18:00 on the fifth day and lasted for 5 days, followed by 5 days of odorant exposure. The odorant bottle was changed for a new one on the third day of odorant exposure. We used >40 MP rats (aged 10–18 months) and selected 35 healthy and normally cyclic rats with aging signals such as shorter period and lower amplitude in circadian rhythm (χ^2 periodogram). Among them, 28 MP rats were selected for exposure to green odor (14 rats) and α-pinene (14 rats). For statistical analysis, the paired *t*-test was applied. All experimental procedures followed *Guidelines for the Care and Use of Laboratory Animals*, Dokkyo University School of Medicine.

Results

Effects of odorants on sleep

Amounts of each sleep–wakefulness stage were calculated every 2 h by manual measuring using 7 day records. The two-way ANOVA revealed no significant differences in mean 2 h amounts of each sleep–wakefulness stage over 24 h all day (06:00–06:00) or over 12 h of daytime (06;00–18:00) between the control and odorant-treated rats. There was no significant difference in alertness and SWS during nighttime (18:00–06:00) between the vehicle and odorant-treated rats, however, the odorant-treated rats showed significant increase in the duration of PS at the 22:00–0:00 period compared with the vehicle-treated rats (Table 1).

Table 1 Effects of odorants on 2 h amounts of SWS and PS during night-time

	Clock time	No.	Vehicle [mean ± SEM (s)]	α-Pinene [mean ± SEM (s)]	Vehicle [mean ± SEM (s)]	Green mix [mean ± SEM (s)]
SWS	18:00	4	2945 ± 401	2678 ± 376	4178 ± 552	3635 ± 46
	20:00	4	1853 ± 528	1750 ± 182	4508 ± 298	3132 ± 567
	22:00	4	3213 ± 89	3670 ± 335	4395 ± 159	3878 ± 677
	0:00	4	2975 ± 755	2628 ± 194	4703 ± 725	4778 ± 524
	2:00	4	2968 ± 281	3503 ± 142	4398 ± 781	2713 ± 500
	4:00	4	1095 ± 552	1430 ± 524	5150 ± 85	5150 ± 804
Two-way ANOVA			$F = 0.26$, $P = 0.62$, not significant		$F = 4.04$, $P = 0.060$, not significant	
PS	18:00	4	375 ± 90	308 ± 44	435 ± 115	268 ± 88
	20:00	4	165 ± 70	128 ± 43	143 ± 82	255 ± 110
	22:00	4	213 ± 16	575 ± 61***	108 ± 27	563 ± 58**
	0:00	4	225 ± 80	375 ± 45	128 ± 56	255 ± 69
	2:00	4	230 ± 43	210 ± 49	165 ± 58	295 ± 64
	4:00	4	88 ± 61	103 ± 37	128 ± 74	165 ± 134
Two-way ANOVA			$F = 5.21$, $P = 0.035$, significant		$F = 5.34$, $P = 0.033$, significant	

Bonferroni post-tests compared with vehicle. $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 2 Odorant effects on circadian rhythmicity

	No. of animals	Mean amplitude of χ^2 periodogram	Mean periods
Vehicle	14	1099 ± 126.2	23.87 ± 0.161
Green mix	14	1561 ± 115.7***	24.20 ± 0.079
Paired *t*-test		$t = 6.86$, $P < 0.0001$	$t = 1.747$, $P = 0.104$, n.s.
Vehicle	14	4488 ± 698	24.16 ± 0.147
α-pinene	14	5888 ± 882.7**	24.74 ± 0.411
Paired *t*-test		$t = 3.04$, $P = 0.0095$	$t = 1.469$, $P = 0.166$, n.s.

$^{**}P < 0.01$; $^{***}P < 0.001$.

Effects of odorants on circadian locomotor rhythm activity

Circadian locomotor rhythms were measured using 10 day records within several-month rhythm records in aged (10–18 months) MP rats. Five days exposure to both odors induced a significant increase in circadian amplitude in χ^2 periodograms. On the other hand, both odors slightly increased the circadian period, but there was no significant difference (Table 2).

Discussion

In the first experiment, a significant increase in the 2 h appearance of PS was observed during the night-time zone (22:00–00:00) in adult SD male rats exposed to plant-derived odorants. The rats dominantly sleep during daytime, but they have small peaks of SWS and PS during the same time zone without any treatment. We have reported similar phenomena in ovariectomized female rats, orchiectomized male rats, septally lesioned rats and aged rats which showed te marked night-time PS peaks (Yamaoka, 1978, 1980, 1983).

MP rats, a congenital mutant rat with growth inhibition of the optic cup (Kobayashi and Otani, 1981), showed an increase in amplitude of circadian activity in χ^2 periodograms during 5 days of exposure to odors. MP rats showed regular free-running circadian rhythm without entraining by the light–dark cycle (Shim *et al.*, 1997). It is well known that aging decreases circadian amplitude and shortens the circadian period. The aging effects of MP rats also started from ~10 months of age and the rhythmicity was almost disrupted by ~24 months of age (data not shown). Amir *et al.* (1999) postulated that olfactory stimulation by cedar wood essence enhanced light-induced phase shift of wheel-running rhythms and suprachiasmatic Fos expression. These findings suggest that these plant-derived odors stimulate olfactory cues and modulate circadian rhythm.

Our previous reports showed that estradiol-treated OVX rats and rats with posterior deafferentation of the hypothalamus increased the amplitude of circadian SWS and PS rhythms (Yamaoka, 1978, 1980, 1983). The present studies and our previous reports may suggest that green odor and α-pinene accelerate PS and ameliorate aging effects, in which some neuroendocrine mechanisms may be involved.

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