

# The Effect of Vapor of Propylene Glycol on Rats

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## Abstract

Propylene glycol (PG) is commonly used as a solvent for odorous chemicals employed in studies of the olfactory system because PG has been considered to be odorless for humans and other animals. However, if laboratory rats can detect the vapor of PG and if exposure to this influences behaviors, such effects might confound data obtained from experiments exposing conscious rats to odorants dissolved in PG. Therefore, we examined this issue using differences in the acoustic startle reflex (ASR) as an index. We also conducted a habituation/dishabituation test to assess the ability of rats to detect the vapor of PG. In addition, we observed  $\text{Ca}^{2+}$  responses of vomeronasal neurons (VNs) in rats exposed to PG using the confocal  $\text{Ca}^{2+}$ -imaging approach. Pure PG vapor significantly enhanced the ASR at a dose of  $1 \times 10^{-4}$  M, which was much lower than the dose for efficiently detecting. In  $\text{Ca}^{2+}$  imaging, VNs were activated by PG at a dose of  $1 \times 10^{-4}$  M or lower. These results suggest that PG vapor acts as an aversive stimulus to rats at very low doses, even lower than those required for its detection, indicating that we should consider such effect of PG when it is employed as a solvent for odorants in studies using conscious rats. In addition, our study suggests that some non-pheromonal volatile odorants might affect animal behaviors via the vomeronasal system.

**Key words:** acoustic startle reflex, aversive stimulus, calcium imaging, habituation/dishabituation test, non-pheromonal odorant, olfaction

## Introduction

According to International Chemical Safety Cards, propylene glycol (1,2-propanediol; PG) is a colorless, viscous, hygroscopic, and essentially odorless liquid dihydric alcohol. Because PG is generally recognized as safe for humans and animals, it is utilized in a variety of fields; for example, PG represents a major constituent of coolants and airplane deicers/anti-icers, a component in hydraulic brake fluids and inks, and a widely used food and cosmetic additive (LaKind et al. 1999; Ishiwata et al. 2003).

Although PG is also used as a vehicle for parenteral administration of hydrophobic drugs, it has been shown that PG itself produces an anxiolytic-like effect and inhibits the central nervous system in ways similar to ethanol (Hanzlik et al. 1939; Zaroslinski et al. 1971; Singh et al. 1982; Lin et al. 1998). Moreover, it has been reported that these effects increase the sedative effect of diazepam in humans (Forrest and Galletly 1988) and the hypnotic effect of pentobarbital in rodents (Singh et al. 1982). These findings suggest that we

should remain alert to the intrinsic pharmacological effects of PG when it is employed as a vehicle for injection in psychopharmacological studies.

In addition, PG has been commonly used as a solvent for odorous chemicals in human experiments examining the olfactory system because PG is considered to be odorless (Schwartz et al. 1994; Fernandez et al. 1999; Kaneda et al. 2000; Kareken et al. 2004; Cashion et al. 2006). For similar reasons, odorous samples were dissolved with PG in some odor-related experiments in rats, including those exploring olfactory discrimination learning (Schoenbaum et al. 1998; Gallagher et al. 1999; Herzog and Otto 2002; Schoenbaum and Setlow 2003; Beaudin et al. 2007). However, if rats can detect vapor of PG, and if exposure to the PG vapor induces some psychogenic effects on behaviors in rats, as observed after parenteral administration of PG, such effects may confound data obtained from experiments exposing conscious rats to odorants dissolved in PG. Therefore, we considered it necessary to assess whether PG vapor influences behaviors of rats because this issue remains unsettled.

Toward this end, this study examined the effect of PG vapor on rats. We investigated differences in the acoustic startle reflex (ASR) in rats exposed to PG vapor (Experiment 1) because the ASR has a nonzero baseline and can be enhanced by aversive and attenuated by rewarding stimuli in rats (reviewed in Koch 1999). We also conducted a habituation/dishabituation test to assess the ability of rats to detect PG vapor (Experiment 2). In addition, we observed  $\text{Ca}^{2+}$  responses of vomeronasal neurons (VNs) to PG in slice preparations of the vomeronasal organ (VNO) using the confocal  $\text{Ca}^{2+}$ -imaging approach to investigate the sensitivity of rat VNs to PG (Experiment 3).

## Materials and methods

### Animals

We purchased 72 experimentally naive male Wistar rats (Clea Japan) at 7 weeks of age for Experiments 1 and 2. During the first week after purchase, they were housed in pairs in wire-topped, transparent cages ( $410 \times 250 \times 180$  mm) with wood shavings for bedding. Next, each rat was housed individually in a new cage of the same type for 1 day before the experimental procedures were initiated. In Experiment 3, 8 male Wistar rats (6–12 weeks old) were used for  $\text{Ca}^{2+}$  imaging. The animals were provided with water and food ad libitum and kept on a 12-h light:dark cycle with the lights turned off at 20:00. The vivarium was maintained at a constant temperature ( $24^\circ\text{C}$ ) and humidity (40–45%). This study was approved by the Animal Care and Use Committee of the Faculty of Agriculture, The University of Tokyo, and by the Kochi Medical School Animal Care and Use Committee; it also conforms to international guidelines on the ethical use of animals. All efforts were made to minimize the number of experimental animals and their suffering.

### Storage of chemicals

We used PG (Wako Pure Chemical Industries) and a structural isomer of PG, 1,3-propanediol (1,3-PD; Wako Pure Chemical Industries), in this study. These chemicals were kept inside regular bottles of Wako Pure Chemical Industries (opaque brown glass bottles; PG: 500 mL, 1,3-PD: 50 mL), each cap of which was sealed with several sheets of Parafilm (American National Can). All bottles containing PG or 1,3-PD were stored in the chemical storage closet under room temperature ( $20\text{--}25^\circ\text{C}$ ). All experiments were performed within 1 year after purchase of chemicals.

### Experiment 1: changes in the ASR in rats exposed to PG

The startle apparatus and software used in this study (StartleReflexSystem 2004; O'Hara & Co.) were described in detail in our previous study (Inagaki et al. 2008). In summary, we used an animal holder to obtain ASR data from each subject rat. The holder consisted of an acrylic cylinder ( $200 \times 60$  mm, 56 mm diameter, 2 mm thickness), front and rear stoppers (acrylic plates,  $100 \times 45$  cm, 2 mm thickness), and an acrylic bottom sheet ( $230 \times 120$  mm, 2 mm thickness) to support the cylinder. The subject rat was kept inside the cylinder using the 2 stoppers, the front of which had a total of 42 perforations (2 mm in diameter). The animal holder was fixed on a platform in a soundproof test chamber ( $480 \times 350 \times 370$  mm) during experiments. Startle responses were elicited by 105-dB, 100-ms white noise auditory stimuli delivered through a high-frequency speaker located on the ceiling of the test chamber, 150 mm above the top of the animal holder. All auditory stimuli were made by an interface (WP-1020; O'Hara & Co.) under the control of the software on a personal computer (OptiPlex GX270; Dell). Background noise (70-dB wideband) was produced by a speaker located at the rear of the soundproof chamber ceiling. Animal movements in the holder resulted in the displacement of an accelerometer (linearity:  $\pm 5\%$  FS) affixed to the bottom of the platform. The voltage output of the accelerometer was digitized and recorded with personal computer software. The startle amplitude was defined as the maximal peak-to-peak voltage that occurred during the first 200 ms after the onset of the startle-eliciting auditory stimulus. A calibration system was used to ensure comparable startle magnitudes across experiments. The test chamber was equipped with 2 white fluorescent bulbs (10 W each) on the ceiling.

All subjects were handled in an experimental room (temperature:  $22^\circ\text{C}$ , humidity: 50–55%) for 5 min and were habituated to the animal holder for 5 min/day beginning 2 days before the experiment. Experiments were conducted for 2 consecutive days; each sample or control stimulus (see below) was presented to subject rats in counterbalanced order. On the day of the experiment, subjects were moved to the experimental room and kept in their home cages for about 60 min before the experiment. The animal was then placed inside the animal holder and fixed on the platform in the

soundproof test chamber. The experiment consisted of 3 procedures: the baseline trial, the sample presentation, and the test trial. During the baseline trial, the subject was first acclimatized for 5 min (under only background noise) and exposed to the 20 auditory stimuli, which were separated by interstimulus intervals of 30 s. Immediately following the baseline trial, the animal holder containing the subject was moved outside the test chamber and a sheet of qualitative filter paper (folded in two, 50 × 50 mm, grade: 2, quality: 100; Toyo Roshi Kaisha) was placed across the perforated front animal stopper at a distance of 10 mm from the rat's nose, enabling the rat to sniff the filter paper without direct contact. Each sample or control stimulus (600 µL) was dropped onto the paper. After 1–2 min of the sample presentation procedure, we returned the animal holder to its place on the platform in the test chamber. Each subject was then exposed to 30 auditory stimuli separated by interstimulus intervals of 30 s in the test trial after the 5-min acclimation period. The filter paper containing each sample or control stimulus was left at a distance of 10 mm from the rat during the test trial.

Presented samples were diluted PG and 1,3-PD. We used 48 subject rats, divided into 4 groups based on dose and chemical, that is,  $1 \times 10^{-2}$  M PG ( $n = 10$ ),  $1 \times 10^{-4}$  M PG ( $n = 14$ ),  $1 \times 10^{-6}$  M PG ( $n = 10$ ), and  $1 \times 10^{-4}$  M 1,3-PD ( $n = 14$ ). All samples were diluted with purified water immediately after production using a water purifier (Auto pure WR600G; Yamato Scientific Co.). To mix solutions, we used unused disposable pipette tips (MultiFit Pipette Tips 100–1000 µL; Sorenson BioScience) and glass beakers (50 mL) washed with a cleaner in an ultrasonic cleaning machine (UT-305S; Sharp Manufacturing Systems) and completely rinsed with purified water. All control samples were made by the same manner using purified water except for each chemical.

### Experiment 2: the habituation/dishabituation test to PG

Each experiment was conducted in the home cage of each subject rat in the vivarium. Before initiation of experimental procedures, the home cage with the subject was moved onto a shelf in the vivarium. After a 1-min acclimation period, each subject rat was exposed to 4 consecutive stimuli. First, a sheet of filter paper (50 × 50 mm, the same used in Experiment 1) soaked with purified water (600 µL) was indirectly presented to the subject rat 10 mm far from the wire ceiling of its home cage using stainless mesh (55 × 55 mm, 480 perforations of 1.5 mm diameter) for 2 min. The subject rat received 2 additional consecutive 2-min presentations of purified water (600 µL each), followed by a 2-min presentation of each PG sample (600 µL, see below), at 30-s intervals, each using a new sheet of filter paper and the same stainless mesh. The behavior of each subject rat was recorded by video (DCR-SR300; Sony) for later analyses.

We used 24 subject rats, divided into 3 groups based on dosages of the PG samples presented, that is, 1 M PG ( $n = 8$ ),  $1 \times 10^{-2}$  M PG ( $n = 8$ ), and  $1 \times 10^{-4}$  M PG ( $n = 8$ ).

PG was solved with purified water using the same manner and materials as we performed in Experiment 1.

### Experiment 3: Ca<sup>2+</sup> imaging of rat VNs in response to PG

Vomeronasal slices were prepared from 6- to 12-week-old Wister rats that had been anesthetized with diethyl ether and decapitated. Methods used in previous studies (Taniguchi et al. 1996; Muramoto et al. 2006) were modified and adapted for the present study. After the vomeronasal neuroepithelia were removed, the epithelia were embedded in agarose (5%) and placed in ice-cold normal Ringer's solution. Coronal slices, ~275 µm thick, were then cut with a vibrating slicer (model HM650; Microme). The slices were incubated in a holding chamber with fluo-4 NW (Invitrogen) containing Ringer's solution (prepared according to the manufacturer's instructions) for 30–45 min at 35 °C and then transferred into recording chambers filled with normal Ringer's solution.

Intracellular Ca<sup>2+</sup> was monitored with fluo-4 NW using a Zeiss LSM 5 confocal microscopy system (Zeiss) attached to a Zeiss Axiovert 100M inverted microscope fitted with a ×20 objective lens (Zeiss, 440640 Plan-Apochromat, NA 0.8). The excitation wavelength of the argon ion laser was set to 488 nm, and fluorescence images emitted at 510 nm (wavelength) were obtained through the LSM 5 system. Images were acquired at 0.5 Hz with an optical thickness of ~25 µm and analyzed using ad hoc hardware and software. VNs could be discriminated easily on the basis of morphology, location in the slice, and responsiveness to high K<sup>+</sup> solution (50 mM KCl, i.e., 47 mM NaCl in normal Ringer's solution was replaced with KCl). All recordings for Ca<sup>2+</sup> transients were performed at room temperature (22–24 °C).

The recording chamber was continuously perfused with a normal Ringer's solution consisting of (in mM) 125 NaCl, 3 KCl, 1 NaH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, and 15 glucose (pH maintained at 7.4 by saturating with 95% O<sub>2</sub>–5% CO<sub>2</sub>). PG and 1,3-PD were dissolved in normal Ringer's solution for the Ca<sup>2+</sup>-imaging experiment. Male rat urine was diluted 1:100 with the Ringer's solution.

Gravity was used to deliver a constant stream of Ringer's solution from the stimulating tube. Five electrically actuated valves were used to switch normal Ringer's and stimulating solutions. The stimulating tube (~200 µm in diameter) was within ~500 µm of the VN lumen, allowing all cells in the field of view to be transiently superfused. To eliminate a non-specific effect from the mechanical stimulation, a slice was irrigated with normal Ringer's solution at the same flow rate as that of the stimulating solution immediately prior to application of the latter. The concentrations of chemicals were represented as concentrations in the pipette.

### Data analysis

In Experiment 1, we defined individual baseline data as the mean amplitude of the last 10 responses in the baseline trial because we controlled for habituation to the auditory stimuli

in each subject and eliminated their first 10 responses. The test data were defined as the mean amplitude of all responses in the test trial. We calculated the difference in amplitude between the test data ( $T$ ) and the baseline data ( $B$ ) as  $T - B$  for each subject. The calculated differences between sample and control data were statistically compared within each experimental group, divided by dose and sample, using the paired  $t$ -test.

In Experiment 2, the duration of the total investigation time for each sample was recorded using Microsoft Excel-based Visual Basic software. The investigation time was defined as the time spent sniffing in the direction of the stimulus with the rat's nose within 10 mm of the stimulus. Data analyses were performed by an experimenter who did not know each test condition. Differences between investigation times for the third purified water stimulus and each PG sample stimulus were analyzed using paired  $t$ -tests.

In Experiment 3, fluorescence signals were quantified by measuring the mean pixel value of a manually selected somatic area, and the fluorescence change,  $\Delta F/F_0$ , was computed, where  $F_0$  is the mean value for the first 10 frames recorded. We plotted relative magnitudes of the  $\text{Ca}^{2+}$  responses to PG (from 6 neurons) and 1,3-PD (from 8 neurons) to describe dose-response curves; the magnitude of the response to PG at the dose of  $1 \times 10^{-4}$  M was taken as unity in each of these. Statistical comparisons were performed using 2-way repeated measures analysis of variance followed by Student's  $t$ -test to compare  $\text{Ca}^{2+}$  responses to each sample dose. In rodents, immunohistochemical and molecular biological studies have demonstrated the spatially segregated subsets of VNs that form distinct layers in the VN neuroepithelium (Dulac and Axel 1995; Halpern et al. 1995; Berghard and Buck 1996; Jia and Halpern 1996; Herrada and Dulac 1997; Matsunami and Buck 1997; Ryba and Tirindelli 1997). One might expect to see differences in response to PG at different levels of the epithelium. To examine whether VNs with somas at different locations in the epithelium responded to PG differently, the relative location of a soma of the PG-sensitive cells in the neuroepithelium was measured. The basal-apical axis of the neuron layer in the epithelium was defined as 0 (apical) and 100 (basal), that is, the closer the value was to 0, the more apically situated was the soma of the neuron in the receptor cell layer. To analyze the location of VNs responded to PG, we compared relative location scores between VNs that showed positive  $\text{Ca}^{2+}$  responses to PG and those that showed negative (i.e., VNs responded to only high  $\text{K}^+$  solution) using Student's  $t$ -test.

All data are displayed as the mean  $\pm$  standard error. The criterion for statistical significance was  $P < 0.05$  for all comparisons.

## Results

### Experiment 1

PG significantly enhanced the ASR at doses of  $1 \times 10^{-2}$  M ( $t = 2.77, P < 0.05$ ) and  $1 \times 10^{-4}$  M ( $t = 2.68, P < 0.05$ ) but not at

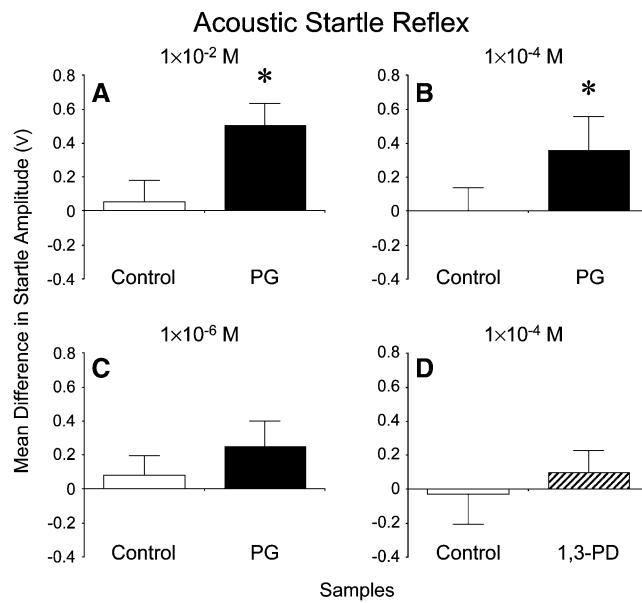
doses of  $1 \times 10^{-6}$  M. However, 1,3-PD did not enhance the ASR significantly at a dose of  $1 \times 10^{-4}$  M (Figure 1).

### Experiment 2

The investigation time gradually decreased through a series of purified water stimuli. The investigation time significantly increased after changing from the third purified water stimulus to the PG sample stimulus at a dose of 1 M ( $t = 3.54, P < 0.01$ ) but not at doses of  $1 \times 10^{-2}$  M ( $t = 0.71, P = 0.50$ ) and  $1 \times 10^{-4}$  M ( $t = 0.88, P = 0.41$ ) (Figure 2).

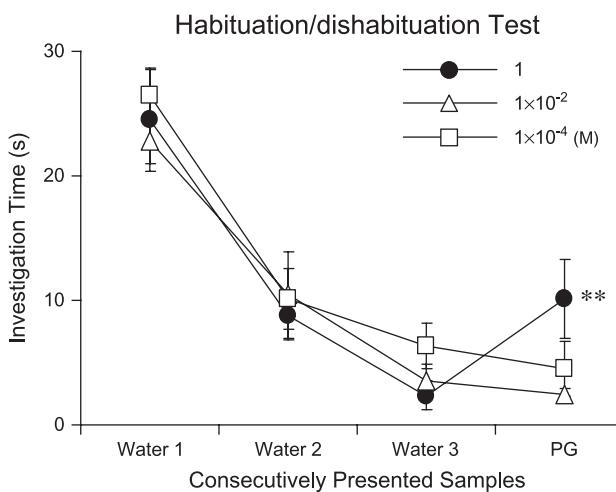
### Experiment 3

Transient  $\text{Ca}^{2+}$  elevations in response to PG were repeatedly observed in the VN with comparable amplitude to urine (100 times diluted) and  $5 \times 10^{-2}$  M KCl solution (Figure 3). Such  $\text{Ca}^{2+}$  responses could be robustly induced by PG at a dose of  $1 \times 10^{-4}$  M but not by PG at a dose of  $1 \times 10^{-5}$  or  $1 \times 10^{-6}$  M (Figure 4A). The amplitudes of the responses to 1,3-PD were much smaller than those of PG at the same concentrations (Figure 4B). In total, we imaged 494 VNs, of which 14 cells (2.8%) responded to  $1 \times 10^{-4}$  M PG. On the other hand, 8 of 466 VNs (1.7%) displayed  $\text{Ca}^{2+}$  transient in response to  $1 \times 10^{-4}$  M 1,3-PD. The dose-response curve (Figure 5) indicates that PG elicited  $\text{Ca}^{2+}$  activations in a dose-dependent manner and that the detection threshold of the VNs for PG was significantly lower than that for 1,3-PD, that is,



**Figure 1** Differences in amplitude between the baseline and test data for the ASR. Subject rats were presented with sample or control (purified water) stimuli in counterbalanced order between the baseline trial and the test trial during 2 consecutive experimental days. Samples presented were PG at doses of  $1 \times 10^{-2}$  M ( $n = 10$ , **A**),  $1 \times 10^{-4}$  M ( $n = 14$ , **B**), and  $1 \times 10^{-6}$  M ( $n = 10$ , **C**), and 1,3-PD at a dose of  $1 \times 10^{-4}$  M ( $n = 14$ , **D**). Each bar represents the mean  $\pm$  standard error;  $*P < 0.05$  versus control (paired  $t$ -test).

statistical analyses indicated a significant effect of sample dose,  $F_{2,24} = 44.2$ ,  $P < 0.01$ ; a significant effect of difference in sample presentation,  $F_{1,12} = 46.5$ ,  $P < 0.01$  ( $1 \times 10^{-4}$  M:  $t = 5.72$ ,  $P < 0.01$ ;  $1 \times 10^{-5}$  M:  $t = 4.02$ ,  $P < 0.01$ ); and a significant interaction between sample presentation and dose,  $F_{2,24} = 15.0$ ,  $P < 0.01$ . The mean relative location of the neurons that responded or did not respond to PG was  $62.2 \pm 6.1$  and  $62.9 \pm 1.1$ , respectively. There was no significant regional difference between the neurons that responded and those that did not respond to PG.



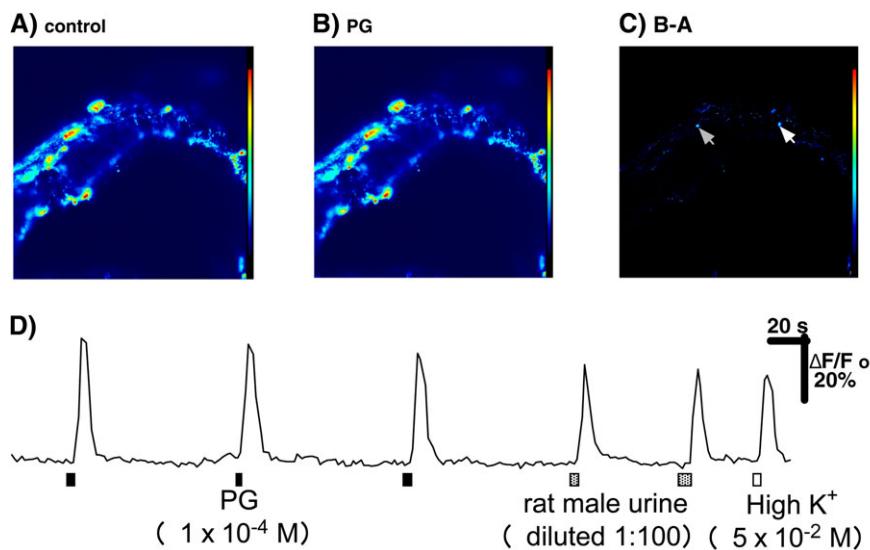
**Figure 2** The investigation time for 3 consecutive water stimuli (Water 1–3) and the following PG stimuli: 1 M ( $n = 8$ ),  $1 \times 10^{-2}$  M ( $n = 8$ ), and  $1 \times 10^{-4}$  M ( $n = 8$ ). Each bar represents the mean  $\pm$  standard error; \*\* $P < 0.01$  versus the third water stimulus (paired  $t$ -test).

Of 71 imaged VNs that were stimulated with both PG ( $1 \times 10^{-4}$  M) and rat male urine (100 times diluted), 4 cells responded to both stimuli, 7 cells responded to only PG, and 60 cells responded only to urine.

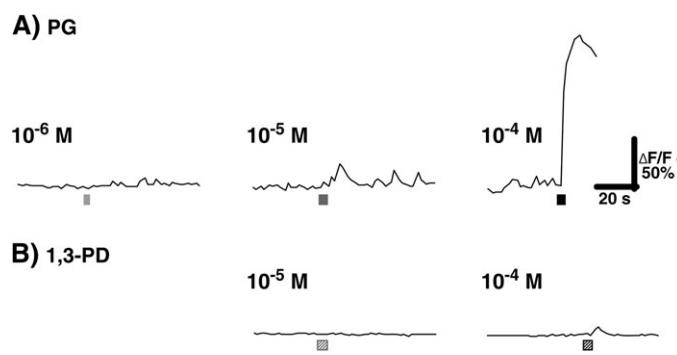
## Discussion

Several lines of evidence obtained in this study indicate that PG vapor is detected and can influence behavior of rats. In Experiment 1, exposure to PG vapor enhanced the ASR in subject rats. In Experiment 2, subject rats used significantly more time to investigate PG vapor than the third water stimulus. This finding indicates that rats could detect PG vapor because earlier studies have demonstrated that a novel odor presented after a successively presented familiar odor causes a longer investigation time for rats and hamsters if the new stimulus can be discriminated from the previous stimulus (Johnston et al. 1993; Kiyokawa et al. 2007). In Experiment 3, VNs in slice preparations of VNOs showed dose-dependent elevations of transient  $\text{Ca}^{2+}$  in response to PG in confocal  $\text{Ca}^{2+}$  imaging. This suggests that VNs were able to detect PG as a ligand because earlier studies of mice have demonstrated similar activation of VNs by some pheromones (Leinders-Zufall et al. 2000; Sam et al. 2001).

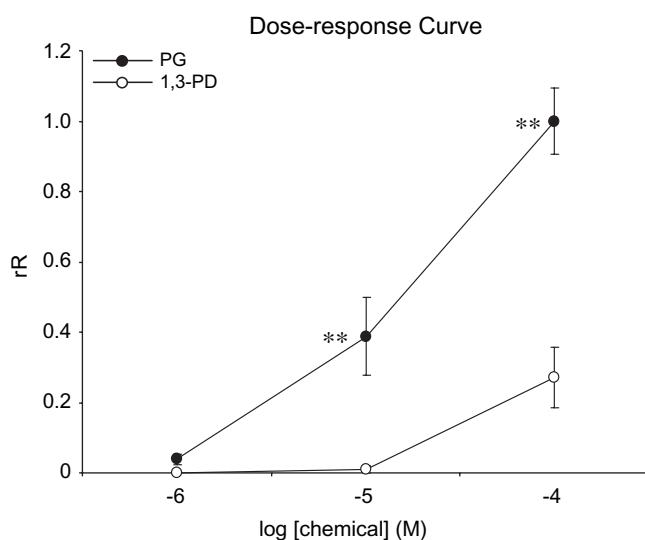
The sensitization of ASR in this study suggested that subject rats experienced an aversive event through exposure to PG vapor. One explanation of this phenomenon is that PG vapor evoked an anxiety-like state in rats via the olfactory system. This view is supported by earlier studies showing that some common odorants elicit anxiety-related responses in rodents at their first exposure to the odor; for example, chocolate powder induced avoidance and risk assessment



**Figure 3**  $\text{Ca}^{2+}$  responses induced by PG in VNs. (A) A confocal fluorescence image acquired at rest (pseudocolor scale); (B) a confocal image acquired just after application of PG to the bath; (C) an image made by subtracting image A from image B, indicating that a limited distribution of VNs respond to PG (arrows); and (D) changes in fluorescence intensity recorded from a VN marked by a gray arrow in image C. Bars below traces indicate the periods in which the indicated stimuli were applied.



**Figure 4** Changes in fluorescence intensity recorded from VNs in response to increasing concentrations of PG (A) and 1,3-PD (B). Bars below traces indicate the periods in which the indicated stimuli were applied.



**Figure 5** Dose dependency of stimulus-induced  $\text{Ca}^{2+}$  responses of VNs that recognized either PG (closed circle) or 1,3-PD (open circle). Relative magnitudes of the responses ( $rR$ ) to PG and 1,3-PD were plotted as a function of chemical concentration. The magnitude of the response to  $1 \times 10^{-4}$  M PG was taken as unity. Each point is the mean  $\pm$  standard error obtained from 6 neurons for PG and 8 neurons for 1,3-PD; \*\* $P < 0.01$  versus relative response to 1,3-PD at the corresponding concentration (2-way repeated measures analysis of variance followed by Student's *t*-test). Note the difference in detection thresholds.

behaviors in mice (Kemble and Gibson 1992; Garbe et al. 1993); cinnamon powder induced avoidance, hypoalgesia, risk assessment behaviors, and suppression of appetitive behaviors in mice (Kemble and Gibson 1992); citronella oil induced hypoalgesia in rats (Lester and Fanselow 1985); and the odor of sheep wool induced risk assessment behaviors and suppression of appetitive behaviors in mice (Garbe et al. 1993; Kemble and Bolwahnn 1997). The other explanation is that PG vapor acted as a chemical agent to cause ocular and/or upper airway irritation. In an inhalation study using rats, longtime exposure to high concentration of PG vapor ( $1 \times 10^{-4}$  mol/L air or more, for 6 h/day, and 5 days/week for 90 days) actually caused nasal hemorrhage and ocular dis-

charge in a high proportion of animals (Suber et al. 1989). Thus, in either case, it is suggested that PG vapor is an aversive stimulus to rats to induce enhancement of the ASR.

The results of this study suggest that PG vapor at a relatively low concentration elicits enhancement of the ASR in rats as efficiently as 2,5-dihydro-2,4,5-trimethylthiazole (TMT), a main chemical contributing to the odor of fox feces. It has been reported that TMT is effective for inducing behavioral and autonomic antipredator responses in rats; for example, previous studies have reported enhancement of the ASR (Endres et al. 2005), freezing behavior (Hotsenpiller and Williams 1997; Wallace and Rosen 2000, 2001; Fendt et al. 2003), avoidance behavior (Burwash et al. 1998; Holmes and Galea 2002), reduced eating (Burwash et al. 1998), and stress-related endocrinological activation (i.e., elevated corticosterone levels) (Vernet-Maury et al. 1984; Morrow et al. 2000, 2002; Soares et al. 2003; Day et al. 2004). In regard to effective concentrations, Endres et al. have demonstrated that the threshold for the TMT-induced freezing behavior was  $3.87 \times 10^{-8}$  mol TMT; in our present study, at least  $6 \times 10^{-8}$  mol PG significantly enhanced the ASR. Although various common agents other than PG have been reported as inducing anxiety-like responses in rodents upon their first exposure, the concentrations of such agents were much higher than that of PG in this study, namely, undiluted or at most a 0.1% solution (Lester and Fanselow 1985; Kemble and Gibson 1992; Garbe et al. 1993; Kemble and Bolwahnn 1997). Moreover, we observed no significant enhancement of the ASR associated with exposure to 1,3-PD, the structural isomer of PG, at the same dose as that used for PG. Therefore, PG vapor may be much more efficient than odors commonly used in evoking behavioral changes in rats.

In this study, although PG vapor enhanced the ASR in Experiment 1 (Figure 1) and incrementally increased investigation time in Experiment 2 (Figure 2) equally and in a dose-dependent manner, the threshold dose of PG in the former ( $1 \times 10^{-4}$  M) was much lower than that in the latter (1 M). These results show that PG vapor can induce sensitization of the ASR in rats at much lower doses than those required for detection using the habituation/dishabituation test. Although this outcome may be simply ascribed to the relative insensitivity of the habituation/dishabituation test to low concentration of odorants compared with the ASR, this phenomenon may be explained by the participation of 2 different olfactory systems in the perception of PG vapor in rats. It is generally assumed that common odorants are detected by the main olfactory epithelium and pheromones are sensed through the VNO. However, studies using a  $\text{Ca}^{2+}$ -imaging technique have demonstrated that non-pheromonal common odorants elicit responses from individual VNs in mice (Sam et al. 2001) and that VNs are much more sensitive than main olfactory neurons to low concentrations of both pheromones and common odorants (Malnic et al. 1999; Leinders-Zufall et al. 2000; Sam et al. 2001). In addition, an earlier study using

high-resolution functional magnetic resonance imaging reported the simultaneous activation of main and accessory olfactory bulbs by non-pheromonal common odors in mice (Xu et al. 2005). Therefore, both the main olfactory system and the vomeronasal system might be related to the generation of the sensitization of the ASR evoked by PG vapor in rats observed in this study. This interpretation is supported by the results of Experiment 3 showing the activation of VNs in response to lower doses of PG ( $1 \times 10^{-4}$  M or less) than those of 1,3-PD in confocal  $\text{Ca}^{2+}$  imaging; however, further studies are necessary to clarify this issue.

In rodents, there are 2 distinct layers in the vomeronasal sensory epithelium (Dulac and Axel 1995; Halpern et al. 1995; Berghard and Buck 1996; Jia and Halpern 1996; Herrada and Dulac 1997; Matsunami and Buck 1997; Ryba and Tirindelli 1997). In mice, it is generally believed that the VNs at the upper layer tend to receive volatile pheromones such as 2-heptanone (Boschat et al. 2002), 6-hydroxy-6-methyl-3-heptanone, *n*-pentyl acetate, and isobutylamine (Del Punta et al. 2002), whereas those at the lower layer tend to detect proteanous pheromones such as exocrine gland-secreting peptide 1 (Kimoto et al. 2005), major histocompatibility complex class I peptides (Leinders-Zufall et al. 2004), and major urinary protein (Chamero et al. 2007). Analysis of relative location of somas in vomeronasal slices in the present study, however, could not determine whether PG induces  $\text{Ca}^{2+}$  transient in VNs at the upper and/or lower layers. One possible reason is that cells responded to PG were located near the border of these 2 layers, which makes it difficult to detect the difference in the locations through the present analysis. There may be another possibility that PG induces  $\text{Ca}^{2+}$  transient in VNs at both layers, although it is less likely than the former possibility. Further investigation will be needed to address this issue.

In general, a given chemical is detected by only a small percentage (less than several percentage) of VNs and its sensitivity profiles were narrowly tuned (Leinders-Zufall et al. 2000). In the present study, relatively a large percentage of VNs (4 of 71 imaged VNs) showed responses to both PG and rat male urine. We do not think, however, that our present results are inconsistent with these observations because of the following reasons. First, it is reported that VNs did respond to multiple ligands. The mouse VNs, for instance, can respond to 2 types of peptide ligands (Leinders-Zufall et al. 2004). In that study, 8 cells of 53 imaged cells that responded to at least one peptide showed  $\text{Ca}^{2+}$  transients to another peptide ligand. Second, urine obviously contains many chemicals including pheromones, which may cause overlapping responsiveness of the VNs between PG and urine.

In conclusion, the results of our study suggest that PG vapor acts as an aversive stimulus to rats at a lower concentration than that required for detection, indicating that investigators should forewarn such effects of PG when it is employed as a solvent for odorants in studies using conscious

rats, particularly in psychopharmacological studies, even if PG appears to be odorless to rats. In addition, our study suggests the possibility that some non-pheromonal volatile odorants may affect the behavior of rats via the vomeronasal system.

## References

Beaudin SA, Stangle DE, Smith DR, Levitsky DA, Strupp BJ. 2007. Succimer chelation normalizes reactivity to reward omission and errors in lead-exposed rats. *Neurotoxicol Teratol.* 29:188–202.

Berghard A, Buck LB. 1996. Sensory transduction in vomeronasal neurons: evidence for G alpha o, G alpha i2, and adenylyl cyclase II as major components of a pheromone signaling cascade. *J Neurosci.* 16:909–918.

Boschat C, Pelofi C, Randin O, Roppolo D, Luscher C, Broillet MC, Rodriguez I. 2002. Pheromone detection mediated by a V1r vomeronasal receptor. *Nat Neurosci.* 5:1261–1262.

Burwash MD, Tobin ME, Woolhouse AD, Sullivan TP. 1998. Laboratory evaluation of predator odors for eliciting an avoidance response in roof rats (*Rattus rattus*). *J Chem Ecol.* 24:49–66.

Cashion L, Livermore A, Hummel T. 2006. Odour suppression in binary mixtures. *Biol Psychol.* 73:288–297.

Chamero P, Marton TF, Logan DW, Flanagan K, Cruz JR, Saghatelian A, Cravatt BF, Stowers L. 2007. Identification of protein pheromones that promote aggressive behaviour. *Nature.* 450:899–902.

Day HEW, Masini CV, Campeau S. 2004. The pattern of brain c-fos mRNA induced by a component of fox odor, 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), in rats, suggests both systemic and processive stress characteristics. *Brain Res.* 1025:139–151.

Del Punta K, Leinders-Zufall T, Rodriguez I, Jukam D, Wysocki CJ, Ogawa S, Zufall F, Mombaerts P. 2002. Deficient pheromone responses in mice lacking a cluster of vomeronasal receptor genes. *Nature.* 419:70–74.

Dulac C, Axel R. 1995. A novel family of genes encoding putative pheromone receptors in mammals. *Cell.* 83:195–206.

Endres T, Apfelbach R, Fendt M. 2005. Behavioral changes induced in rats by exposure to trimethylthiazoline, a component of fox odor. *Behav Neurosci.* 119:1004–1010.

Fendt M, Endres T, Apfelbach R. 2003. Temporary inactivation of the bed nucleus of the stria terminalis but not of the amygdala blocks freezing induced by trimethylthiazoline, a component of fox feces. *J Neurosci.* 23:23–28.

Fernandez M, Schwartz GER, Bell IR. 1999. Subjective ratings of odorants by women with chemical sensitivity. *Toxicol Ind Health.* 15:577–581.

Forrest P, Galletly DC. 1988. A double-blind comparative-study of 3 formulations of diazepam in volunteers. *Anaesth Intensive Care.* 16: 158–163.

Gallagher M, McMahan RW, Schoenbaum G. 1999. Orbitofrontal cortex and representation of incentive value in associative learning. *J Neurosci.* 19:6610–6614.

Garbe CM, Kemble ED, Rawleigh JM. 1993. Novel odors evoke risk assessment and suppress appetitive behaviors in mice. *Aggress Behav.* 19:447–454.

Halpern M, Shapiro LS, Jia C. 1995. Differential localization of G proteins in the opossum vomeronasal system. *Brain Res.* 677:157–161.

Hanzlik PJ, Newman HW, VanWinckle W. Jr, Lehman AJ, Kennedy NK. 1939. Toxicity, fats and excretion of propylene glycol and some other glycols. *J Pharmacol Exp Ther.* 67:101–113.

Herrada G, Dulac C. 1997. A novel family of putative pheromone receptors in mammals with a topographically organized and sexually dimorphic distribution. *Cell.* 90:763–773.

Herzog CD, Otto T. 2002. Administration of transforming growth factor-alpha enhances anatomical and behavioral recovery following olfactory nerve transection. *Neuroscience.* 113:569–580.

Holmes MM, Galea LAM. 2002. Defensive behavior and hippocampal cell proliferation: differential modulation by naltrexone during stress. *Behav Neurosci.* 116:160–168.

Hotzenpiller G, Williams JL. 1997. A synthetic predator odor (TMT) enhances conditioned analgesia and fear when paired with a benzodiazepine receptor inverse agonist (FG-7142). *Psychobiology.* 25: 83–88.

Inagaki H, Kiyokawa Y, Kikusui T, Takeuchi Y, Mori Y. 2008. Enhancement of the acoustic startle reflex by an alarm pheromone in male rats. *Physiol Behav.* 93:606–611.

Ishiwata H, Nishijima M, Fukasawa Y. 2003. Estimation of inorganic food additive (nitrite, nitrate and sulfur dioxide), antioxidant (BHA and BHT), processing agent (propylene glycol) and sweetener (sodium saccharin) concentrations in foods and their daily intake based on official inspection results in Japan in fiscal year 1998. *J Food Hyg Soc Jpn.* 44:132–143.

Jia C, Halpern M. 1996. Subclasses of vomeronasal receptor neurons: differential expression of G proteins (Gi alpha 2 and G(o alpha)) and segregated projections to the accessory olfactory bulb. *Brain Res.* 719:117–128.

Johnston RE, Derzie A, Chiang G, Jernigan P, Lee HC. 1993. Individual scent signatures in golden-hamsters: evidence for specialization of function. *Anim Behav.* 45:1061–1070.

Kaneda H, Maeshima K, Goto N, Kobayakawa T, Ayabe-Kanamura S, Saito S. 2000. Decline in taste and odor discrimination abilities with age, and relationship between gustation and olfaction. *Chem Senses.* 25: 331–337.

Kareken DA, Sabri M, Radnovich AJ, Claus E, Foresman B, Hector D, Hutchins GD. 2004. Olfactory system activation from sniffing: effects in piriform and orbitofrontal cortex. *Neuroimage.* 22:456–465.

Kemble ED, Bolwahnn BL. 1997. Immediate and long-term effects of novel odors on risk assessment in mice. *Physiol Behav.* 61:543–549.

Kemble ED, Gibson BM. 1992. Avoidance and hypoalgesia induced by novel odors in mice. *Psychol Rec.* 42:555–563.

Kimoto H, Haga S, Sato K, Touhara K. 2005. Sex-specific peptides from exocrine glands stimulate mouse vomeronasal sensory neurons. *Nature.* 437:898–901.

Kiyokawa Y, Kikusui T, Takeuchi Y, Mori Y. 2007. Removal of the vomeronasal organ blocks the stress-induced hyperthermia response to alarm pheromone in male rats. *Chem Senses.* 32:57–64.

Koch M. 1999. The neurobiology of startle. *Prog Neurobiol.* 59:107–128.

LaKind JS, McKenna EA, Hubner RP, Tardiff RG. 1999. A review of the comparative mammalian toxicity of ethylene glycol and propylene glycol. *Crit Rev Toxicol.* 29:331–365.

Leinders-Zufall T, Brennan P, Widmayer P, Chandramani P, Maul-Pavicic A, Jager M, Li XH, Breer H, Zufall F, Boehm T. 2004. MHC class I peptides as chemosensory signals in the vomeronasal organ. *Science.* 306: 1033–1037.

Leinders-Zufall T, Lane AP, Puche AC, Ma WD, Novotny MV, Shipley MT, Zufall F. 2000. Ultrasensitive pheromone detection by mammalian vomeronasal neurons. *Nature.* 405:792–796.

Lester LS, Fanselow MS. 1985. Exposure to a cat produces opioid analgesia in rats. *Behav Neurosci.* 99:756–759.

Lin HQ, Burden PM, Johnston GAR. 1998. Propylene glycol elicits anxiolytic-like responses to the elevated plus-maze in male mice. *J Pharm Pharmacol.* 50:1127–1131.

Malnic B, Hirono J, Sato T, Buck LB. 1999. Combinatorial receptor codes for odors. *Cell.* 96:713–723.

Matsunami H, Buck LB. 1997. A multigene family encoding a diverse array of putative pheromone receptors in mammals. *Cell.* 90:775–784.

Morrow BA, Elsworth JD, Roth RH. 2002. Fear-like biochemical and behavioral responses in rats to the predator odor, TMT, are dependent on the exposure environment. *Synapse.* 46:11–18.

Morrow BA, Redmond AJ, Roth RH, Elsworth JD. 2000. The predator odor, TMT, displays a unique, stress-like pattern of dopaminergic and endocrinological activation in the rat. *Brain Res.* 864:146–151.

Muramoto K, Huang GZ, Taniguchi M, Kaba H. 2006. Functional synapse formation between cultured rat accessory olfactory bulb neurons and vomeronasal pockets. *Neuroscience.* 141:475–486.

Ryba NJ, Tirindelli R. 1997. A new multigene family of putative pheromone receptors. *Neuron.* 19:371–379.

Sam M, Vora S, Malnic B, Ma WD, Novotny MV, Buck LB. 2001. Odorants may arouse instinctive behaviours. *Nature.* 412:142.

Schoenbaum G, Chiba AA, Gallagher M. 1998. Orbitofrontal cortex and basolateral amygdala encode expected outcomes during learning. *Nat Neurosci.* 1:155–159.

Schoenbaum G, Setlow B. 2003. Lesions of nucleus accumbens disrupt learning about aversive outcomes. *J Neurosci.* 23:9833–9841.

Schwartz GE, Bell IR, Dikman ZV, Fernandez M, Kline JP, Peterson JM, Wright KP. 1994. EEG responses to low-level chemicals in normals and cacosmics. *Toxicol Ind Health.* 10:633–643.

Singh PP, Junnarkar AY, Seshagirirao C, Kaushal R, Naidu MUR, Varma RK, Tripathi RM, Shridhar DR. 1982. A pharmacological study of propane-1,2-diol. *Arzneimittelforschung.* 32:1443–1446.

Soares DD, Fernandez F, Aguerre S, Foury A, Mormede P, Chaouloff F. 2003. Fox odour affects corticosterone release but not hippocampal serotonin reuptake and open field behaviour in rats. *Brain Res.* 961:166–170.

Suber RL, Deskin R, Nikiforov I, Fouillet X, Coggins CRE. 1989. Subchronic nose-only inhalation study of propylene-glycol in Sprague-Dawley rats. *Food Chem Toxicol.* 27:573–583.

Taniguchi M, Kashiwayanagi M, Kurihara K. 1996. Intracellular dialysis of cyclic nucleotides induces inward currents in turtle vomeronasal receptor neurons. *J Neurosci.* 16:1239–1246.

Vernet-Maury E, Polak EH, Demael A. 1984. Structure-activity relationship of stress-inducing odorants in the rat. *J Chem Ecol.* 10:1007–1018.

Wallace KJ, Rosen JB. 2000. Predator odor as an unconditioned fear stimulus in rats: elicitation of freezing by trimethylthiazoline, a component of fox feces. *Behav Neurosci.* 114:912–922.

Wallace KJ, Rosen JB. 2001. Neurotoxic lesions of the lateral nucleus of the amygdala decrease conditioned fear but not unconditioned fear of a predator odor: comparison with electrolytic lesions. *J Neurosci.* 21:3619–3627.

Xu FQ, Schaefer M, Kida I, Schafer J, Liu N, Rothman DL, Hyder F, Restrepo D, Shepherd GM. 2005. Simultaneous activation of mouse main and accessory olfactory bulbs by odors or pheromones. *J Comp Neurol.* 489:491–500.

Zarolsinski JF, Browne RK, Possley LH. 1971. Propylene glycol as a drug solvent in pharmacologic studies. *Toxicol Appl Pharmacol.* 19:573–578.