J Chem Ecol (2017) 43:263–272
DOI 10.1007/s10886-017-0822-3

Structure-Activity Relationships of Alkylpyrazine Analogs and Fear-Associated Behaviors in Mice

Kazumi Osada[1] · **Sadaharu Miyazono**[2] · **Makoto Kashiwayanagi**[2]

Received: 20 December 2016 / Revised: 24 January 2017 / Accepted: 26 January 2017 / Published online: 28 February 2017

Abstract Our previous studies identified alkyl pyrazine analogs in wolf urine that act as novel kairomones and induce a series of fear-associated behaviors in mice. A mixture of these alkyl pyrazines also effectively suppressed the approach of deer to a feeding area, and animals that did approach the marked area exhibited fear-associated behaviors. To investigate structure-activity relationships of alkyl pyrazines, four fear-associated behaviors - freezing, locomotion activity, odor investigation, and avoidance - were measured in experiments on female C57BL/6 J mice. Of the 17 compounds tested, 2,3-diethylpyrazine, 3-ethyl-2,5-dimethylpyrazine, and 3-ethyl-2,5-dimethylpyrazine induced all four fear-associated behaviors. 2,3,5-Trimethylpyrazine also induced three of the fear-associated behaviors, but did not decrease locomotion. Multivalent analysis of behaviors clearly demonstrated that these four compounds formed an independent cluster and were the most active. Structure-activity relationships revealed that active alkyl pyrazines inducing all four fear-associated behaviors had methyl or ethyl group(s), but not longer carbon chains, and alkyl side chains consisting of four carbon atoms in total were present in the most potent analogs. This study is the first experimental investigation of structure-activity relationships between alkyl pyrazine analogs and fear-associated behaviors in mice.

✉ Kazumi Osada
osadak@hoku-iryo-u.ac.jp

[1] Division of Physiology, Department of Oral Biology, School of Dentistry, Health Sciences University of Hokkaido, Tobetsu, Ishikari, Hokkaido 061-0293, Japan

[2] Department of Sensory Physiology, Asahikawa Medical University, Asahikawa, Hokkaido, Japan

Keywords Alkyl pyrazine analogs · Wolf · Fear-associated behavior · Kairomone · Structure-activity relationship

Introduction

The common gray wolf (*Canis lupus*) is found throughout the entire Northern hemisphere and preys on various mammals including deer, rabbits, squirrels, and mice. The wolf is a gregarious carnivore that mainly utilizes chemo-olfactory modes of communication (Fox and Cohen 1977). Wolf urine induces avoidance behavior in cattle (Kluever et al. 2009), wild animals (Parsons and Blumstein 2010; Severud et al. 2011; Sullivan et al. 1985a, b), and laboratory rats (Fendt 2006).

We previously reported that odors in the urine of the gray wolf induce aversive and fear-associated responses in mice in an experimental setting (Osada et al. 2013). These responses are mainly caused by the presence of certain volatile pyrazine compounds, namely, 2,6-dimethylpyrazine (2,6-DMPz), 2,3,5-trimethylpyrazine (2,3,5-TMPz), and 2,5-dimethyl-3-ethylpyrazine (3-EDMPz). A recent study by Brechbühl et al. (2015) also demonstrated that some pyridine and pyrazine analogs, including those described above, were present in the urine of mountain lion (*Puma concolor*). In other studies by our group (Osada et al. 2014, 2015), Hokkaido deer (*Cervus nippon yesoensis*) were found to be repelled by a cocktail of the alkyl pyrazines in wolf urine, and this odor significantly inhibited their approach to a feeding area. Additionally, vigilance behaviors such as tail-flagging, flight, and jumping were significantly higher in the presence of this pyrazine cocktail, and the effects lasted at least one month after commencement of the experiment.

There are several examples of alkyl pyrazine analogues that act as semiochemicals in plants (Bohman et al. 2012, 2014), insects (Derstine et al. 2012; Nilsson and Bengtsson 2004;

Sharma et al. 2011; Tentschert et al. 2000; Vander Meer et al. 2010; Yu et al. 2014), terrestrial vertebrates (Daev et al. 2012; Jemiolo and Novotny 1994; Novotny et al. 1986; Woolfson and Rothschild 1990; Zhang et al. 2005), and marine organisms (Dickschat et al. 2005). Therefore, it is conceivable that there may be additional, as-yet-unidentified alkyl pyrazine analogues that trigger fear-associated responses in mice. Although several semiochemicals may be potentially toxic (Kaufman et al. 2010; Kumar and Gautam 2008; Poiner et al. 2007; Tierney et al. 2010), these alkyl pyrazine analogues are not expected to be toxic. Pyrazine analogs contribute to the characteristic roasted aromas in various foods where they are safe for human consumption, they are non-carcinogenic, and they have low acute toxicity (Burdock and Carabin 2008; EFSA Panel on Food Contact Materials and (CEF). 2011). Alkyl pyrazine analogs may therefore function as effective kairomones for manipulating the behavior of herbivores without destroying natural habitats or disrupting agriculture.

To investigate the structure-activity correlation between 17 alkyl pyrazine analogs, we investigated four different types of fear-associated behavior in mice: duration of freezing, innate investigation of the odor source, locomotor activity, and avoidance of each pyrazine analog. We aimed to determine the chemical structures of the most active alkyl pyrazine analogs, and to identify novel compounds that induce fear-associated behaviors more effectively than naturally occurring pyrazines present in wolf urine.

Methods and Materials

Experimental Animals Mice were cared for in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The Animal Ethics and Research Committee of the Health Sciences University of Hokkaido approved the experimental protocols prior to initiation of the study (approval ID: 059).

Experimental animals were female C57BL/6 J mice. Animals were kept in a room maintained at 22 °C with a photoperiod of 12 h:12 h (non-reversed 12 h light/dark cycle). Mice were housed in polycarbonate cages (two to three animals per cage) in a sterile animal facility and provided with ad libitum access to a standard murine diet (Lab Chow, MF, Oriental Yeast, Tokyo, Japan) and water.

Odorants for Behavioral Tests Seventeen alkylpyrazines (**1**–**17**) with different alkyl substituents (Table 1) were tested for their ability to stimulate fear-associated behaviors in mice. In addition, four analogs (**18**–**21,** Fig. 1) of 2,3-diethylpyrazine (**14)** were tested to explore the effect of a 3-ethyl group on fear-associated behaviors. All chemicals were of analytical grade and were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) or Alfa Aesar (Lancashire, United Kingdom).

Compounds were diluted to 10% with distilled water (**1**–**17**) or diethyl phthalate (Wako Pure Chemical Industries, LTD, Osaka, Japan) (**18**–**21**) because the latter test compounds were not completely soluble in water alone. We therefore included each of the solvents in control experiments. These solvents induced very weak fear-associated behaviors. For example, there were no significant differences in freezing duration between water (17.1 ± 2.5 s/10 min), diethyl phthalate (21.0 ± 4.2 s/10 min), and herbivore (*Oryctolagus cuniculus*) urine (22.8 ± 2.7 s/10 min) (N = 6 per group).

Fear-Associated Behavioral Tests in an Open Arena A total of 144 sexually inexperienced, 2–5 month old female mice (N = 6 per group) served as test animals and were exposed to 17 pyrazine analogues (**1**–**17,** Table 1). All behavioral studies were performed as blind tests. To avoid complicating the data due to learning effects, mice were only tested once. To explore the fear-inducing properties of the alkyl pyrazines, freezing-in-place, odor source investigation, and locomotor activity assessments were conducted simultaneously in a rectangular, open-bottomed polycarbonate chamber (27 × 17 × 14 cm). An aluminum exhaust duct (11 cm diameter) attached to the polyethylene mesh on the inlet was connected from the upper center of the chamber to the exhaust port in the experimental room. This experimental chamber was connected to an Animex activity meter Model DSE (LKB, Muromachi Kikai Co., Ltd. Tokyo, Japan). Mice were first familiarized to the chamber by placing them inside for 5 min on each of two consecutive days. On the third day, individual alkyl pyrazine analogs (10 μl of 10% *v*/v) were applied to pieces of filter paper (2.5 × 2.5 cm), placed into Petri dishes (35 mm diameter), and inserted into the chamber of the Animex activity meter.

All animal behavior was recorded with a video camera (Ivis HF R42, Canon, Tokyo, Japan), and the investigation time for filter paper tests was 5–10 min. An animal was considered to be investigating the odor source when its snout was oriented towards the filter paper and held within 1 cm of it (Osada et al. 2008). Freezing (immobilization) duration was measured, and freezing was defined as adopting a crouched posture with no body movement other than that essential for breathing (Endres and Fendt 2009). Locomotor activity was measured and quantified using an Animex activity meter. Mice were placed on top of the meter, and each movement produced a signal due to variations in inductance and capacity of the apparatus resonance circuit. The sensitivity of the activity meter was adjusted to record mainly locomotion. These signals were automatically converted to numerical data.

Y-Maze Avoidance Behavior To confirm the above results in a different experimental setting, independent Y-maze

Table 1 Compounds tested in the behavioral experiments

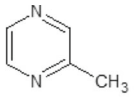
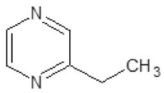
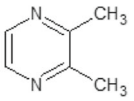
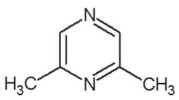
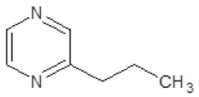
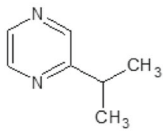
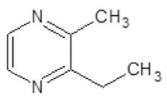
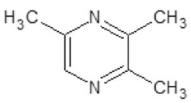
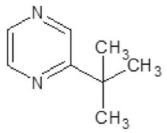
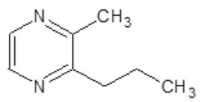
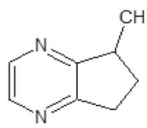
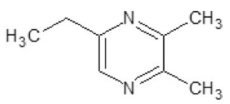
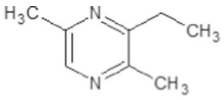
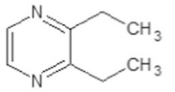
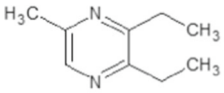
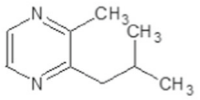
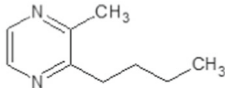
#	Structure	Name (abbr.)	Carbon Atom(s)[a]	Vapor Pressure (mmHg at 25°C)[b]
1		2-methylpyrazine (2-MPz)	1	9.7±0.2
2		2-ethylpyrazine (2-EPz)	2	4.0±0.3
3		2,3-dimethylpyrazine (2,3-DMPz)	2	3.4±0.3
4		2,6-dimethylpyrazine (2,6-DMPz)	2	3.9±0.2
5		2-propylpyrazine (2-PPz)	3	1.4±0.3
6		2-isopropylpyrazine (2-IPPz)	3	2.0±0.3
7		2-ethyl-3-methyl pyrazine (2-E-3-MPz)	3	1.7±0.3
8		2,3,5-trimethylpyrazine (2,3,5-TMPz)	3	1.7±0.3
9		2-tert-butylpyrazine (2-t-BPz)	4	1.2±0.3
10		2-propyl-3-methyl pyrazine (2-P-3-MPz)	4	0.8±0.3
11		6,7-dihydro-5H-5-methyl-cyclopentapyrazine (5-M-CPPz)	4	0.3±0.4
12		2,3-dimethyl-5-ethyl pyrazine (5-EDMPz)	4	0.7±0.4

Table 1 (Continued)

13		2,5-dimethyl-3-ethyl pyrazine (3-EDMPz)	4	1.2±0.3
14		2,3-diethylpyrazine (2,3-DEPz)	4	1.0±0.3
15		2,3-diethyl-5-methyl pyrazine (5-M-DEPz)	5	0.5±0.4
16		2-isobutyl-3-methyl pyrazine (2-IB-3-MPz)	5	0.3±0.4
17		2-butyl-3-methyl pyrazine (2-B-3-MPz)	5	0.2±0.4

Chemicals with italic numbers are present in wolf urine (Osada et al. 2013)
[a] Total number of carbon atoms forming the functional group(s) attached to the pyrazine ring
[b] Vapor pressure data were obtained from the Chemspider homepage (http://www.chemspider.com)

avoidance was investigated using sexually inexperienced 2–5-month-old mice ($N = 82$). Test mice ($N > 6$ per group) were assigned to one of 11 alkyl pyrazines, which included nine compounds that induced at least one fear-associated behavior in the open arena tests described above. Again, mice were only tested once to avoid complications from the effects of learning. Avoidance tests were conducted in a blind manner in a custom-made Y-maze (long arm length 450 mm, short arm length 110 mm to the polypropylene gate, arm width 100 mm, airflow 138 ml/min) as described by Osada et al. (2011). Before initiation of the experiment, mice were habituated to the Y-maze for 4 min, and, on the next day, 10 μl of the 10% solution of the test alkyl pyrazine was applied to pieces of filter paper (2.5 × 2.5 cm), placed into Petri dishes, and inserted into the short arm of the Y-maze. Filter papers of the same size but spotted with water (10 μl) served as controls and were simultaneously inserted into the other short arm of the Y-maze. Animals were then inserted into a long arm of the Y-maze. To prevent direct contact between mouse noses and filter papers, polypropylene gates with holes (1 cm diameter) were placed between them.

Thus, test mice were denied almost all sensory information except for olfactory-mediated stimulation from the odor source. Animal behavior was recorded with a video camera as above. The amount of time spent in each of the short arms of the Y-maze was measured over a period of 4 min. Odor sources were inserted at random into either short arm. Each test was conducted between 12:00 noon and 17:00 h. The floor of the test area was replaced with a clean bench coat (Whatman protector sheets Cat No. 2300 916, Whatman, Maidstone, UK) between each trial to eliminate residual odorant cues. The avoidance rate was taken to be the amount of time spent in the short arm containing the control odor divided by the total amount of time spent in both short arms (control vs. pyrazine analog), multiplied by 100. Water was substituted for pyrazine analogs in control experiments (control vs. control).

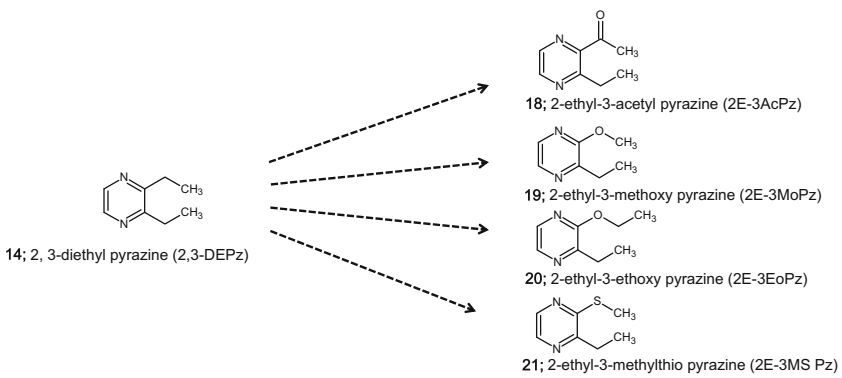


Fig. 1 Analogs of 2,3-diethylpyrazine (2,3-DEPz) tested in behavioral experiments

Statistical Analysis Data are given as means ± SEM. The statistical significance of differences elicited by each of the pyrazine analogs was assessed by ANOVA followed by Dunnett's post-hoc test. In addition, the Pearson r value was used for determining the statistical significance of the correlation analysis between fear-associated behaviors and distance measurements. Hierarchical cluster analysis was performed using the average linkage method for squared Euclidian distances, and shows relatively homogeneous clusters of cases based on the characteristics measured. The hierarchical clustering process is represented as a tree in which each step in the clustering process is illustrated by a branch. The horizontal scale corresponds to the linkage distances (LDs) obtained from the analysis.

Results

Relationship between Freezing Duration, Investigation Time, and Locomotor Activity We first analyzed the interrelationship between the three types of fear-associated behavior induced by the 17 alkyl pyrazine analogs (Table 1). There was a significant correlation between freezing duration and locomotor activities (Fig. 2a): the longer the freezing duration of mice confronted with a particular pyrazine analogue, the lower was the locomotor activity detected by the Animex apparatus ($r = -0.907$; $P < 0.001$). A similar relationship was apparent between freezing and investigation duration at both 5 min ($r = -0.375$; $P < 0.001$) and 10 min ($r = -0.380$; $P < 0.001$; Fig. 2b). There was also a positive correlation between sniffing duration and locomotor activity at high sensitivity (data not shown; $r = 0.285$; $P < 0.05$). These results provide clear evidence of a correlation between fear-induced behaviors.

Effects of Alkylpyrazine Analogs on Fear-Associated Behaviors As shown in Fig. 2, ANOVA of the mean time spent frozen (not moving) during 5 and 10 min exposures to each of the alkyl pyrazine analogs revealed a significant effect on freezing behavior (5 min, $F_{17,90} = 4.034$, $P < 0.001$; 10 min, $F_{17,90} = 5.673$, $P < 0.001$). Dunnett's post-hoc tests showed that 2,3,5-TMPz (**8**; $P = 0.002$), 5-EDMPz (**12**; $P = 0.003$), 3-EDMPz (**13**; $P < 0.001$), and 2,3-DEPz (**14**; $P < 0.001$) all had a significant effect on increasing freezing behavior, relative to water controls (Fig. 3).

ANOVA of the mean investigation time revealed that alkyl pyrazine odor had a significant effect by decreasing locomotor activity ($F_{17,90} = 3.339$, $P < 0.001$). Additionally, Dunnett's post-hoc test showed that compounds **12** ($P = 0.023$), **13** ($P = 0.004$), and **14** ($P = 0.005$) had the greatest effect on reducing locomotor activity (Fig. 4), consistent with the freezing results above.

We simultaneously performed innate olfactory preference tests for each of the 17 alkyl pyrazine analogs and the mean investigation times for 5 and 10 min exposures are shown in Fig. 5. ANOVA revealed a significant influence of the compounds on investigation time (5 min, $F_{17,90} = 2.758$, $P < 0.01$; 10 min, $F_{17,90} = 3.979$, $P < 0.001$). Dunnett's post-hoc tests showed that 9 of the 17 compounds were investigated for significantly less time than were the water controls (Fig. 5), namely, **5** ($P = 0.001$), **6** ($P = 0.025$), **7** ($P = 0.013$), **8** ($P < 0.001$), **12** ($P = 0.002$), **13** ($P < 0.001$), **14** ($P < 0.001$), **15** ($P = 0.002$), and **16** ($P < 0.001$).

Y-Maze Avoidance Behavior To confirm the above results in a different experimental setting, we conducted avoidance tests using a Y-maze. ANOVA revealed a significant influence of the compounds on the avoidance rate ($F_{11,62} = 2.806$, $P < 0.01$). Dunnett's post-hoc tests showed that 5 of the 11 alkyl pyrazines (including nine compounds that induced at least one fear-associated behavior in the open arena tests) were significantly avoided by mice. These were **5** ($P = 0.024$), **8** ($P = 0.047$), **12** ($P = 0.035$), **13** ($P = 0.006$), and **14** ($P = 0.004$). These chemicals also induced freezing more strongly than the other compounds (Fig. 3). In addition to these highly active compounds, compound 5 also induced avoidance but not freezing and locomotion activities (Figs. 3 and 4).

Multivalent Analysis of Fear-Associated Behaviors Induced by Alkylpyrazine Analogs Alkylpyrazine analogs (11 out of 17) and the control group were subjected to all four

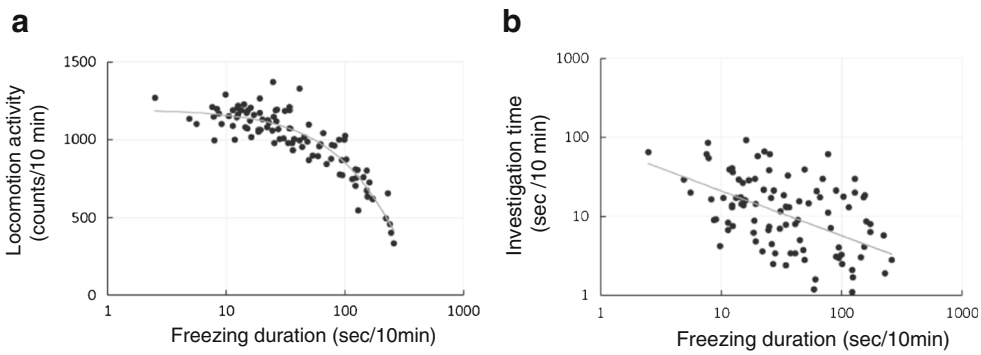


Fig. 2 Correlation between freezing duration and **a** locomotion activities and **b** odor investigation in mice exposed to 17 alkyl pyrazine analogs

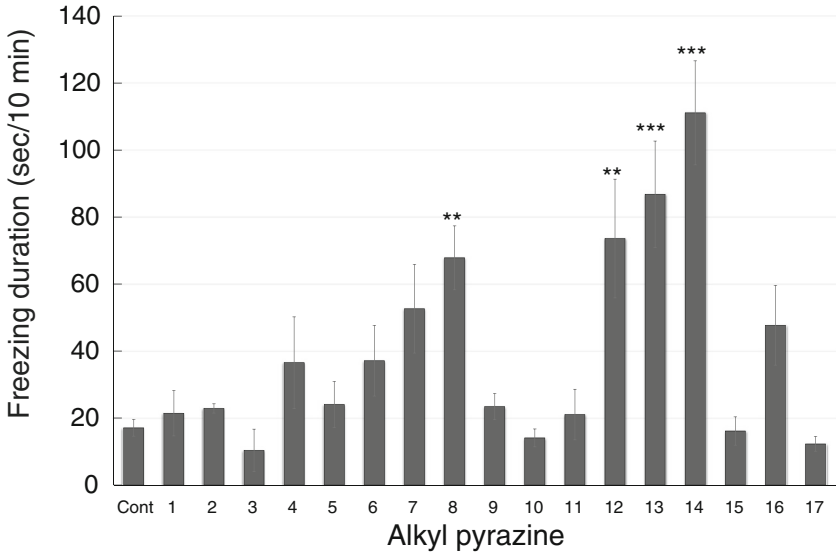


Fig. 3 Comparison of freezing duration in mice exposed to 17 alkyl pyrazine analogues for 10 min. The statistical significance of differences between freezing duration of pyrazine-treated vs. control animals was assessed by ANOVA followed by Dunnett's post-hoc test (**$P < 0.01$, ***$P < 0.001$)

behavioral analyses. In total, 12 groups were subjected to hierarchical cluster analysis of the response of fear-associated behaviors. The results indicated two large clusters (Fig. 7). Initially, branches corresponding to **12**, **13**, and **14** formed one distinct cluster consistent with the fear-associated behavioral data, and 8 was located alone but was part of the same cluster as **12**, **13**, and **14**. The LD between **12**, **13**, **14**, and **8** was 5.0. Secondly, alkyl pyrazines **1**, **5**, **6**, **15**, **16**, and **18** formed a large cluster with the control group, and a small cluster consisting of 4 and 7 formed a neighboring cluster together with the control group (LD = 3.0). The LD between these two large clusters was 25.0.

Behavioural Effects of Analogs of 2,3-Diethylpyrazine (14) The above results indicated that 2,3-DEPz (**14**) was among the most powerful alkyl pyrazine inducers of fear-associated behavior (Figs. 3, 4, 5, 6, and 7). Four additional 2,3-DEPz analogs (**18**–**21**; Fig. 1) were tested in open arena tests and did not induce higher fear-associated responses than **14**. In particular, analog **18** failed to induce any of the three behavioral responses measured. Although less active than **14**, compounds **19**, **20**, and **21** induced at least one fear-associated behavior relative to controls (increased freezing duration: **14**, $P < 0.001$, **20**, $P < 0.05$; reduced investigation time: **14**, $P < 0.001$, **19**, $P < 0.01$, **20**, $P < 0.001$, **21**, $P < 0.01$; reduced locomotion activity: **14**, $P < 0.05$).

Discussion

Induction of Fear-Associated Behaviors by Alkyl Pyrazine Analogs The present study demonstrates that a single application of various pyrazine analogs could induce fear-associated behaviors in C57BL/6j mice. Of the 17 alkyl

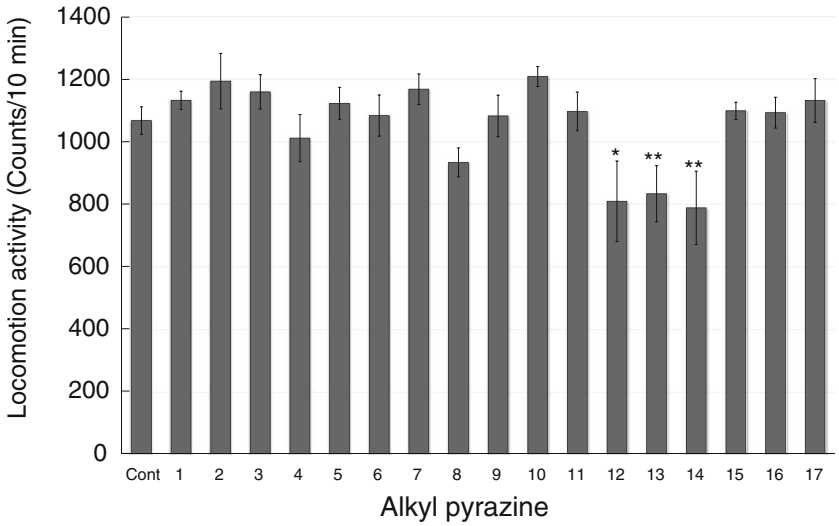


Fig. 4 Comparison of the locomotor activities of mice during 10 min exposure to 17 alkyl pyrazine analogues. The statistical significance of differences between responses to pyrazines vs. controls was assessed by ANOVA followed by Dunnett's post-hoc test (*$P < 0.05$, **$P < 0.01$)

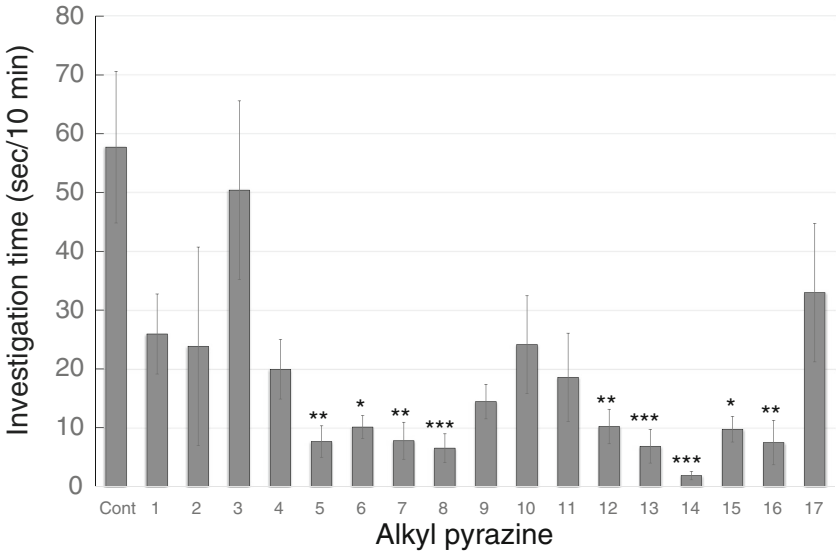


Fig. 5 Comparison of investigation time of mice exposed to 17 alkyl pyrazine analogues for 10 min. The statistical significance of differences between responses to pyrazines vs. controls was assessed by ANOVA followed by Dunnett's post-hoc test (*$P < 0.05$, **$P < 0.01$, *** $P < 0.001$)

pyrazine analogs tested, 5-EDMPz (**12**), 3-EDMPz (**13**), 2,3-DEPz (**14**), and 2,3,5-TMPz (**8**) increased the freezing duration by 436%, 500%, 610%, and 400% compared with control group animals (Fig. 3). Of these four active compounds, **8** and **13** are known kairomones from wolf urine that initiate defensive behavior in mice (Osada et al. 2013). In the present study, we successfully reproduced these findings, then went on to identify novel active pyrazine analogues (**12** and **14**) not found in wolf urine. Compound **14** in particular was equivalent to or even stronger in fear-inducing activity than previously characterized wolf urine kairomones (**8** and **13**). These results demonstrate that pyrazine analogs that are more potent than naturally occurring kairomones can be developed.

We also investigated the effects of the compounds on locomotion activity, and found that **12**, **13**, and **14** significantly decreased this behavior (80%, 82%, and 78% relative to controls), while 8 (86% relative to controls) also had a marginal but not statistically significant effect. These four compounds also elicited a significantly longer freezing duration than was observed in control animals. It is conceivable that this decrease in locomotor activity was at least partly due to an increase in freezing duration. To test this hypothesis, we analyzed the relationship between three different fear-associated behaviors induced by the pyrazine analogues, and confirmed a significant correlation between freezing duration and locomotor activities.

Previous studies demonstrated that trimethylthiazoline (TMT) induces a number of fear-associated responses, including a decrease in vertical and horizontal locomotion activities,

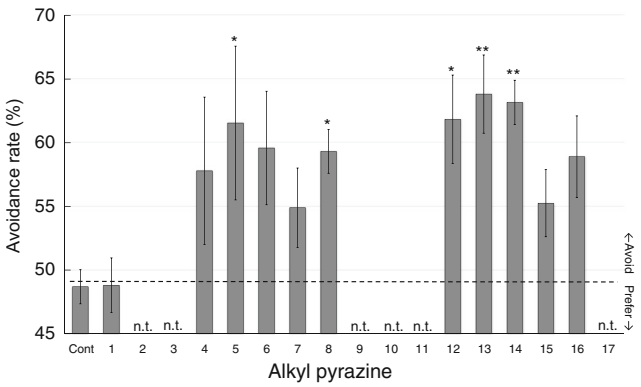


Fig. 6 Avoidance rate of mice exposed to 11 alkylyrazine analogs in a Y-tube olfactometer. The avoidance rate was defined as the amount of time spent in the short arm of the Y-maze containing the control odor (water) divided by the total amount of time spent in both short arms (containing alkyl pyrazine analog and water). The statistical significance of the differences between avoidance rate in response to pyrazines vs. water (controls) was assessed by ANOVA followed by Dunnett's post-hoc test (*$P < 0.05$, **$P < 0.01$). *n.t.* not tested

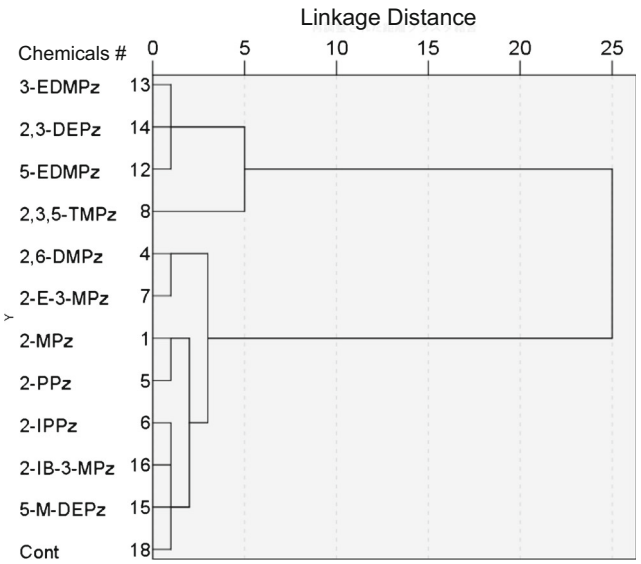


Fig. 7 Multivalent analysis of alkyl pyrazine analogs and fear-associated behaviors. The figure shows a tree diagram obtained from a hierarchical clustering analysis of fear-associated behaviors of mice induced by alkylpyrazine analogues. The *horizontal scale* corresponds to the linkage distances obtained from the hierarchical cluster analysis

and a decrease in the time spent in the center of an open field area (Vernet-Maury et al. 1984). In addition to locomotion activity, freezing behavior is widely used in behavioral studies to indicate fear (Fanselow and Helmstetter 1988; Fendt et al. 2005; Wallace and Rosen 2000). Moreover, recent studies demonstrated that 2-s-butyl-3,4-dihydrothiazole (SBT) and various pyridine analogues could increase freezing duration and decrease walking distance. For example, SBT increased the freezing duration by 188% and the walking distance reduced to 64.4% that of control animals (Brechbühl et al. 2008, 2013). Although the chemical structures of these kairomones are different, the behavioral changes observed in these previous studies are consistent with those observed in the present work. An increase in freezing behavior and a simultaneous decrease in locomotion activity therefore appear to be typical fear-associated behaviors induced by kairomones in mice.

In addition, recent neuro-pharmacological studies indicated a negative correlation between freezing duration and locomotor activity, following pharmacological treatment of mice. For instance, small conductance Ca^{2+}-activated K^+ channel (SK channel) activators produced a transient decline in locomotor behavior and an increase in immobility (Vick et al. 2010). Furthermore, chronic restraint stress facilitates synaptic plasticity in the anterior cingulate cortex via increased excitability due to preventing the inhibition of $GABA_A$ receptor signaling, which may underlie induction of behavioral hyper-locomotive activity and reduced freezing behavior following chronic restraint stress (Ito et al. 2010). Pharmacological studies such as these may help us to understand the perception and behavioral induction mechanisms underlying the effects of kairomones on mice and other prey animals.

In addition to freezing duration and locomotor activity, we assessed the time spent investigating the odor source in an open arena as well as odor avoidance in a Y-tube bioassay. Nine pyrazine analogs, including the four that induced freezing behavior described above, were avoided to a greater extent than the control odor, and all pyrazine analogs were unattractive to mice to some extent. Interestingly, mice avoided investigating 9 out of 17 compounds, whereas only four compounds induced freezing behavior. This finding indicates that a decrease in odor investigation behavior was provoked not only through fear induction, but also through the simple innate avoidance of noxious compounds. Thus, the correlation coefficient between investigation time and freezing duration was significant, but smaller than that between freezing duration and locomotor activities. In a previous study that evaluated the odor investigation behavior of mice in response to kairomones, mice avoided investigating odors not only due to the presence of the fear inducer TMT, but also due to the presence of various noxious chemicals such as butyric acid (Kobayakawa et al. 2007). Together, the results of the innate odor avoidance tests indicate that mice respond sensitively but less specifically to kairomone odors than they do in freezing and locomotor activity tests.

Structure-Activity Relationships of Alkyl Pyrazine Analogs From the results of the behavioral experiments and the multivariate analysis, 2,3-DEPz (**14**), 3-EDMPz (**13**), and 5-EDMPz (**12**) were the three most active alkyl pyrazine analogs. A recent study demonstrated that 2-methylthiazoline (2-MT), a derivative of TMT, induced freezing behavior more than did TMT (Isosaka et al. 2015). The vapor pressure of 2-MT is six times higher than that of TMT, and while the mechanism underlying the neurophysiological potency of 2-MT remains unknown, the higher freezing activity may be attributable to the higher vapor pressure. Similarly, it has been postulated that the relatively low molecular weight and consequent high vapor pressure and gas phase concentration of alkyl pyrazines may be of relevance to their fear-inducing activity (Laska et al. 2009; Sarrafchi et al. 2013). However, the smaller molecular-weight analogs in the present study possessing one or two carbon atoms in their alkyl chains did not induce fear-associated behaviors, suggesting that the fear-inducing activity of alkyl pyrazines is not simply due to their high vapor pressure.

The alkyl side chains of the most potent analogues contained four carbon atoms (**12**, **13**, and **14**; Table 1). Comparing the activity of **14** with **7**, when the ethyl group of **14** was substituted by a methyl group, three of the four fear-associated behaviors were stimulated to a lesser extent. Similarly, comparing the activities of **14** and **15**, attachment of an additional methyl group to generate a 5-carbon chain in **15** did not enhance activity compared with **14**. Moreover, substituting the ethyl group of **12** or **13** to a methyl group in **8** failed to decrease locomotor activity. These results suggest that the total number of carbon atoms in the alkyl chain groups attached to the alkyl pyrazine analogs can have an effect on the induction of fear-associated behaviors.

However, other analogues (**9**, **10**, and **11**) possessing longer alkyl chain groups (propyl, butyl, etc.) did not induce fear-associated behaviors, even when the functional groups consisted of four carbon atoms. Taken together, our results suggest that a pyrazine with one or more methyl or ethyl groups consisting in total of four carbon atoms is necessary for eliciting full fear-associated behavioral activities.

To explore further the structure-activity relationships of alkyl pyrazine analogs, we conducted behavioral studies using 2,3-DEPz (**14**) and analogs with additional acetyl, methoxy, ethoxy, and methylthio groups (**18**–**21**). The results showed that all substituted analogs had a lower fear-inducing activity than compound **14**, suggesting that two ethyl groups attached to **14** may be optimal for inducing fear-associated behavior in mice. However, we cannot preclude the possibility that pyrazine analogues with other substituents may be more powerful

fear inducers, especially since **19**, **20**, and **21** were able to induce fear-associated behaviors in more than one test. Future studies should explore a wider range of substituted alkyl pyrazine analogs.

The neurological mechanisms underlying the potent fear-inducing activity of alkyl pyrazine analogs are not known. Previous studies for other kairomones indicated that TMT and 2-phenylethylamine stimulate the main olfactory bulb (Ferrero et al. 2011; Kobayakawa et al. 2007). Major urinary proteins (MUPs) in rat urine (MUP-13) or cat saliva (MUP Feld4) stimulate the accessory olfactory bulb (Papes et al. 2010), and all induce fear-associated behaviors. Further research is needed to determine the neurophysiological mechanisms involved.

In conclusion, the results of the current study revealed the following: (1) a significant negative correlation between freezing duration and both investigation time and locomotor activity; (2) 5-EDMPz (**12**), 3-EDMPz (**13**), and 2,3-DEPz (**14**) were the most active components inducing fear-associated behaviors, and 2,3-DEPz was even more effective than naturally occurring compounds present in wolf urine; (3) the attachment to pyrazine of one or more methyl or ethyl groups up to a total of four carbon atoms is required to induce full fear-associated behavioral activities. Thus, it is anticipated that these active pyrazine analogs may be employed as novel and powerful repellents for controlling wild animals. Moreover, our approach could be used to explore additional potentially more effective, non-naturally occurring pyrazine kairomones, and further study will be required to clarify their activities in other prey animals.

Acknowledgements This work was supported by the Japanese Society for the Promotion of Science KAKENHI Grant (26450141 and 15 K18573).

References

Bohman B, Jeffares L, Flematti G, Phillips RD, Dixon KW, Peakall R, Barrow RA (2012) The discovery of 2-hydroxymethyl-3-(3-methylbutyl)-5-methylpyrazine: a semiochemical in orchid pollination. Org Lett 14:2576–2578

Bohman B, Phillips RD, Menz MHM, Berntsson BW, Flematti GR, Barrow RA, Dixon KW, Peakall R (2014) Discovery of pyrazines as pollinator sex pheromones and orchid semiochemicals: implications for the evolution of sexual deception. New Phytol 203:939–952

Brechbühl J, Klaey M, Broillet M-C (2008) Grueneberg ganglion cells mediate alarm pheromone detection in mice. Science 321:1092–1095

Brechbühl J, Moine F, Klaey M, Nenniger-Tosato M, Hurni N, Sporkert F, Giroud C, Broillet M-C (2013) Mouse alarm pheromone shares structural similarity with predator scents. Proc Natl Acad Sci U S A 110:4762–4767

Brechbühl J, Moine F, Nenniger Tosato M, Sporkert F, Broillet M-C (2015) Identification of pyridine analogs as new predator-derived kairomones. Front Neurosci 9:253

Burdock GA, Carabin IG (2008) Safety assessment of 2-ethyl-3,(5 or 6) dimethylpyrazine as a food ingredient. Regul Toxicol Pharmacol 50: 303–312

Daev EV, Vyborova AM, Kazarova VE, Dukel'skaya AV (2012) Effect of two pyrazine-containing chemosignals on cells of bone marrow and testes in male house mice (*Mus musculus L.*). J Evol Biochem Phys 48:18–23

Derstine NT, Troyer EJ, Suttles CN, Siderhurst LA, Jang EB, Siderhurst MS (2012) Field trapping the little fire ant, *Wasmannia auropunctata*. J Insect Sci 12:93

Dickschat JS, Wagner-Döbler I, Schulz S (2005) The chafer pheromone buibuilactone and ant pyrazines are also produced by marine bacteria. J Chem Ecol 31:925–947

EFSA Panel on Food Contact Materials Enzymes, Flavourings and Processing Aids (CEF) (2011) Scientific opinion on flavouring group evaluation 17, revision 3 (FGE.17Rev3): pyrazine derivatives from chemical group 24. EFSA J 9:2456–2522

Endres T, Fendt M (2009) Aversion- vs fear-inducing properties of 2, 4, 5-trimethyl-3-thiazoline, a component of fox odor, in comparison with those of butyric acid. J Exp Biol 212:2324–2327

Fanselow MS, Helmstetter FJ (1988) Conditional analgesia, defensive freezing, and benzodiazepines. Behav Neurosci 102:233–243

Fendt M (2006) Exposure to urine of canids and felids, but not of herbivores, induces defensive behavior in laboratory rats. J Chem Ecol 32:2617–2627

Fendt M, Endres T, Lowry CA, Apfelbach R, McGregor IS (2005) TMT-induced autonomic and behavioral changes and the neural basis of its processing. Neurosci Biobehav Rev 29:1145–1156

Ferrero DM, Lemon JK, Fluegge D, Pashkovski SL, Korzan WJ, Datta SR, Spehr M, Fendt M, Liberles SD (2011) Detection and avoidance of a carnivore odor by prey. Proc Natl Acad Sci U S A 108:11235–11240

Fox MW, Cohen JA (1977) Canid communication. In: Sebeok TA (ed) How animals communicate. Indiana University Press, Bloomington, pp 728–748

Isosaka T, Matsuo T, Yamaguchi T, Funabiki K, Nakanishi S, Kobayakawa R, Kobayakawa K (2015) Htr2a-expressing cells in the central amygdala control the hierarchy between innate and learned fear. Cell 163:1153–1164

Ito H, Nagano M, Suzuki H, Murakoshi T (2010) Chronic stress enhances synaptic plasticity due to disinhibition in the anterior cingulate cortex and induces hyper-locomotion in mice. Neuropharmacology 58: 746–757

Jemiolo B, Novotny M (1994) Inhibition of sexual maturation in juvenile female and male mice by a chemosignal of female origin. Physiol Behav 55:519–522

Kaufman PE, Mann RS, Butler JF (2010) Evaluation of semiochemical toxicity to *Aedes aegypti, Ae. albopictus* and *Anopheles quadrimaculatus* (Diptera: Culicidae). Pest Manag Sci 66:497–504

Kluever BM, Howery LD, Breck SW, Bergman DL (2009) Predator and heterospecific stimuli alter behaviour in cattle. Behav Process 81: 85–91

Kobayakawa K, Kobayakawa R, Matsumoto H, Oka Y, Imai T, Ikawa M, Okabe M, Ikeda T, Itohara S, Kikusui T, Mori K, Sakano H (2007) Innate versus learned odour processing in the mouse olfactory bulb. Nature 450:503–508

Kumar G, Gautam N (2008) Allelotoxicity of *Parthenium* leaf extracts on cytomorphological behaviour of sunflower (*Helianthus annuus*). J Environ Biol 29:243–247

Laska M, Persson O, Hernandez Salazar LT (2009) Olfactory sensitivity for alkylpyrazines – a comparative study in CD-1 mice and spider monkeys. J Exp Zool A 311:278–288

Nilsson E, Bengtsson G (2004) Endogenous free fatty acids repel and attract Collembola. J Chem Ecol 30:1431–1443

Novotny M, Jemiolo B, Harvey S, Wiesler D, Marchlewska-Koj A (1986) Adrenal-mediated endogenous metabolites inhibit puberty in female mice. Science 231:722–725

Osada K, Tashiro T, Mori K, Izumi H (2008) The identification of attractive volatiles in aged male mouse urine. Chem Senses 33:815–823

Osada K, Hanawa M, Tsunoda K, Izumi H (2011) Alteration of mouse urinary odor by ingestion of the xenobiotic monoterpene citronellal. Chem Senses 36:137–147

Osada K, Kurihara K, Izumi H, Kashiwayanagi M (2013) Pyrazine analogues are active components of wolf urine that induce avoidance and freezing behaviours in mice. PLoS One 8:e61753

Osada K, Miyazono S, Kashiwayanagi M (2014) Pyrazine analogs are active components of wolf urine that induce avoidance and fear-associated behaviors in deer. Front Behav Neurosci 8:276

Osada K, Miyazono S, Kashiwayanagi M (2015) The scent of wolves: pyrazine analogs induce avoidance and vigilance behaviors in prey. Front Neurosci 9:363

Papes F, Logan DW, Stowers L (2010) The vomeronasal organ mediates interspecies defensive behaviors through detection of protein pheromone homologs. Cell 141:692–703

Parsons MH, Blumstein DT (2010) Familiarity breeds contempt: kangaroos persistently avoid areas with experimentally deployed dingo scents. PLoS One 5:e10403

Poiner GO Jr, Marshall CJ, Buckley R (2007) One hundred million years of chemical warfare by insects. J Chem Ecol 33:1663–1669

Sarrafchi A, Odhammer AME, Hernandez Salazar LT, Laska M (2013) Olfactory sensitivity for six predator odorants in CD-1 mice, human subjects, and spider monkeys. PLoS One 8:e80621

Severud WJ, Belant JL, Bruggink JG, Windels SK (2011) Predator cues reduce American beaver use of foraging trails. Human-Wildl Interact 5:296–305

Sharma K, Vander Meer RK, Fadamiro HY (2011) Phorid fly, *Pseudacteon tricuspis*, response to alkylpyrazine analogs of a fire ant, *Solenopsis invicta*, alarm pheromone. J Insect Physiol 57:939–944

Sullivan TP, Nordstrom LO, Sullivan DS (1985a) Use of predator odors as repellents to reduce feeding damage by herbivores: I. Snowshoe hares (*Lepus americanus*). J Chem Ecol 11:903–919

Sullivan TP, Nordstrom LO, Sullivan DS (1985b) Use of predator odors as repellents to reduce feeding damage by herbivores: II. Black-tailed deer (*Odocoileus hemionus columbianus*). J Chem Ecol 11: 921–935

Tentschert J, Bestmann H-J, Hölldobler B, Heinze J (2000) 2, 3-dimethyl-5-(2-methylpropyl) pyrazine, a trail pheromone component of *Eutetramorium mocquerysi* Emery (1899) (hymenoptera: Formicidae). Naturwissenschaften 87:377–380

Tierney KB, Baldwin DH, Hara TJ, Ross PS, Scholz NL, Kennedy CJ (2010) Olfactory toxicity in fishes. Aquat Toxicol 96:2–26

Vander Meer RK, Preston CA, Choi MY (2010) Isolation of a pyrazine alarm pheromone component from the fire ant, *Solenopsis invicta*. J Chem Ecol 36:163–170

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

Vick KA, Guidi M, Stackman RW Jr (2010) *In vivo* pharmacological manipulation of small conductance Ca(2+)-activated K(+) channels influences motor behavior, object memory and fear conditioning. Neuropharmacology 58:650–659

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

Woolfson A, Rothschild M (1990) Speculating about pyrazines. Proc Biol Sci 242:113–119

Yu Y, Jang EB, Siderhurst MS (2014) Differential field responses of the little fire ant, *Wasmannia auropunctata* (Roger), to alarm pheromone enantiomers. J Chem Ecol 40:1277–1285

Zhang JX, Soini HA, Bruce KE, Wiesler D, Woodley SK, Baum MJ, Novotny MV (2005) Putative chemosignals of the ferret (*Mustela furo*) associated with individual and gender recognition. Chem Senses 30:727–737