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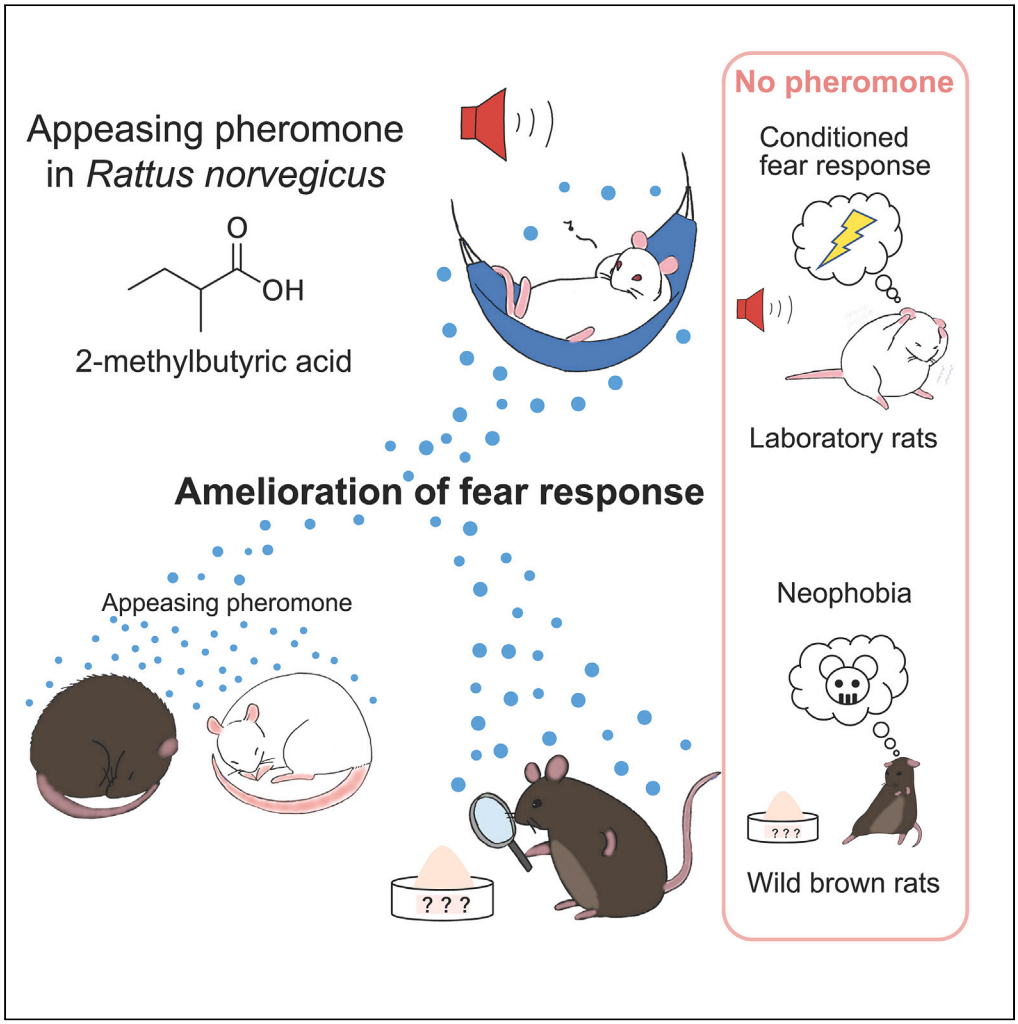

Yasushi Kiyokawa, Shigeyuki Tamogami, Masato Ootaki, ..., Satoru Nagaoka, Tsutomu Tanikawa, Yukari Takeuchi

akiyo@mail.ecc.u-tokyo.ac.jp

Highlights

Both laboratory rats and wild brown rats release 2-methylbutyric acid (2-MB)

2-MB ameliorated conditioned fear responses in laboratory rats

2-MB ameliorated neophobia in wild brown rats

We conclude that 2-MB is an appeasing pheromone in the brown rat, *Rattus norvegicus*

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An appeasing pheromone ameliorates fear responses in the brown rat (*Rattus norvegicus*)

Yasushi Kiyokawa,[1,5,*] Shigeyuki Tamogami,[1] Masato Ootaki,[1] Evelyn Kahl,[2] Dana Mayer,[2] Markus Fendt,[2] Satoru Nagaoka,[3] Tsutomu Tanikawa,[4] and Yukari Takeuchi[1]

SUMMARY

The brown rat (*Rattus norvegicus*) is one of the major animals both in the laboratory and in urban centers. Brown rats communicate various types of information using pheromones, the chemicals that mediate intra-species communication in minute amounts. Therefore, analyses of pheromones would further our understanding of the mode of life of rats. We show that a minute amount of 2-methylbutyric acid (2-MB) released from the neck region can ameliorate fear responses both in laboratory rats and in wild brown rats. Based on these findings, we conclude that 2-MB is an appeasing pheromone in the brown rat. A better understanding of rats themselves would allow us to perform more effective ecologically based research on social skills and pest management campaigns with low animal welfare impacts, which might contribute to furthering the advancement of science and improving public health.

INTRODUCTION

The brown rat (*R. norvegicus*) is one of the major animals both in the laboratory and in urban centers. Over the past decade, the demand for understanding its mode of life is increasing in both places. In the laboratory, there has been an increasing awareness by the neuroscience community that laboratory rats can be used as an animal model for the analysis of social skills.[1] Such analyses would be more effective when the experiments are performed in more ecologically based paradigms. In urban centers, pest control campaigns have been performed for thousands of years. Nonetheless, wild brown rats are still one of the major pests around the world. Therefore, more ecologically based methods are necessary to control wild brown rats more effectively.

Pheromones are olfactory signals that mediate communication between members of the same species in minute amounts.[2] In mammals, pheromones have been considered to transmit information mostly regarding the physiological state of the releaser, e.g., sex, age, or reproduction status.[3] In addition, recent progress in the analyses of pheromones suggests that rats can transmit their stress status using pheromones. For example, distressed rats release a mixture of 4-methylpentanal and hexanal as the main component of alarm pheromone that evokes anxiety responses in other rats.[4] Given that communication plays a pivotal role in the life of social animals, analyses of pheromones would further our understanding of the mode of life of brown rats.

In laboratory rats, the presence of a non-stressed rat can ameliorate fear responses caused by an aversive conditioned stimulus (CS).[5] A series of studies demonstrated that this effect is achieved by the volatile odor detected by the main olfactory epithelium. When the main olfactory epithelium had been lesioned, the rat showed fear responses even in the presence of a non-stressed rat.[6] In contrast, the volatile odor released from a non-stressed rat alone was sufficient to ameliorate fear responses.[7] Based on these findings, we hypothesized that an appeasing pheromone that ameliorates fear responses is present in the volatile odor released from non-stressed rats.

Here, we tried to identify an appeasing pheromone in the brown rat. We first established a method to collect the volatile odor containing the appeasing pheromone, i.e., the bioactive odor, from anesthetized rats. Comparisons between bioactive and non-bioactive odors showed that four chemicals were shared by all bioactive odors, but were absent in non-bioactive odors. Subsequent analyses using synthetic chemicals showed that a minute amount of 2-methylbutyric acid (2-MB), but not the other three

[1]Laboratory of Veterinary Ethology, the University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

[2]Institute for Pharmacology and Toxicology & Center for Behavioral Brain Sciences, Otto-von-Guericke University Magdeburg, D-39120 Magdeburg, Germany

[3]Daimaru Compound Chemical Co., Ltd, Nagano 381-1222, Japan

[4]Technical Research Laboratory, Ikari Shodoku Co., Ltd, Chiba 275-0024, Japan

[5]Lead contact

*Correspondence: akiyo@mail.ecc.u-tokyo.ac.jp

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candidate chemicals, was as effective as the bioactive odor. In addition, 2-MB and the bioactive odor produced the same pattern of neural activity when ameliorating conditioned fear responses. Furthermore, when presented to non-stressed rats, 2-MB had a rewarding value. Given that laboratory rats and wild brown rats are members of the same species, the effectiveness of 2-MB in wild brown rats was further explored. It was found that a substantial proportion of wild brown rats released 2-MB. In addition, 2-MB ameliorated neophobia, an avoidance of novel objects, both in the laboratory and in the field. Taken together, it was concluded that 2-MB acts as an appeasing pheromone that ameliorates fear responses in the brown rat (*R. norvegicus*).

RESULTS

Establishing a method to collect bioactive odors in laboratory rats

To identify the appeasing pheromone in laboratory rats, it is necessary to collect a large amount of volatile odor that contains the appeasing pheromone, i.e., the bioactive odor. However, distressed rats are known to release an alarm pheromone that augments anxiety responses.[4] Therefore, if the collection method stresses the donor rat, the presence of an appeasing pheromone cannot be appropriately evaluated, because the alarm pheromone will be released simultaneously. To circumvent this issue, we first aimed to establish a method to collect bioactive odors from anesthetized donor rats. During our studies on the alarm pheromone, we found that electrical stimulation of the skin of anesthetized rats induced muscle contractions, which enhanced the release of body odor from the stimulated region.[8] We also demonstrated that water could be used as a carrier of the released odor from the anesthetized donor rat.[9] Taking advantage of these findings, volatile odor samples were prepared by stimulation of the head, neck, flank, or rump region of anesthetized Wistar donor rats. Water was used as a carrier of these samples in a bioassay where fear-conditioned Wistar rats were exposed to an auditory CS in the presence of the odor sample to be assessed. It was found that the neck odor, and the head odor to some degree, ameliorated conditioned fear responses, including freezing behavior (Figures 1A and S1). This could have been due to the stronger intensity of neck odor. When the odor-containing water was diluted 2-fold, rats could only detect the presence of neck odor in water (Figure 1B). To the best of our knowledge, there are no specific scent glands in the neck region. Therefore, it is more probable that we are collecting general body odor. Given that one of the sources of body odor is sebum produced in the sebaceous glands, we assessed whether the sebaceous glands are more abundant in the neck region than in the other body regions. However, these glands were not specifically abundant in the neck region (Figure 1C). Therefore, differences in the abundance of sebaceous glands are less likely to account for the stronger intensity of neck odor. Taken together, these results suggest that the bioactive odor can be collected most effectively from the neck region of anesthetized rats.

One methodological approach to identify the appeasing pheromone is to focus on the chemicals that are shared by all bioactive odors, but are absent in non-bioactive odors. The conditioned fear response in Wistar rats is known to be ameliorated by the presence of non-stressed Wistar and Sprague-Dawley (SD) rats, but not by the presence of non-stressed Fischer344 (F344) or Brown Norway (BN) rats.[10] Therefore, a suitable set of bioactive and non-bioactive odors can be prepared from these rats. The bioassay showed that the neck odor of Wistar and SD rats, but not the neck odor of F344 rats, ameliorated conditioned fear responses, including freezing behavior (Figures 1D and S2). The effectiveness of the neck odor of BN rats was unclear (Figure 1D). These results suggest that neck odors released from Wistar, SD, and F344 rats can be used as a suitable set of bioactive and non-bioactive odors for identifying appeasing pheromones.

2-MB functions as an appeasing pheromone that ameliorates fear responses in laboratory rats

To identify the candidate chemicals for an appeasing pheromone, the components of neck odor from each of seven Wistar, SD, and F344 rats were individually analyzed. The neck regions of anesthetized rats were stimulated electrically, and air was drawn around the neck region through a glass tube containing Tenax, an adsorbent that can trap a wide range of volatile chemicals (Figures 2A and S3). Analysis of the samples prepared from Wistar rats by gas chromatography and mass spectrometry (GC-MS) showed that all chromatograms shared 63 chemicals. Of them, 17 chemicals were apparently environmental contaminants, e.g., benzene, phenol, etc. Therefore, 46 chemicals were the focus of study. The chemical profile of each sample was obtained by calculating the content percentage of each chemical with respect to these 46 chemicals (Figure S4).

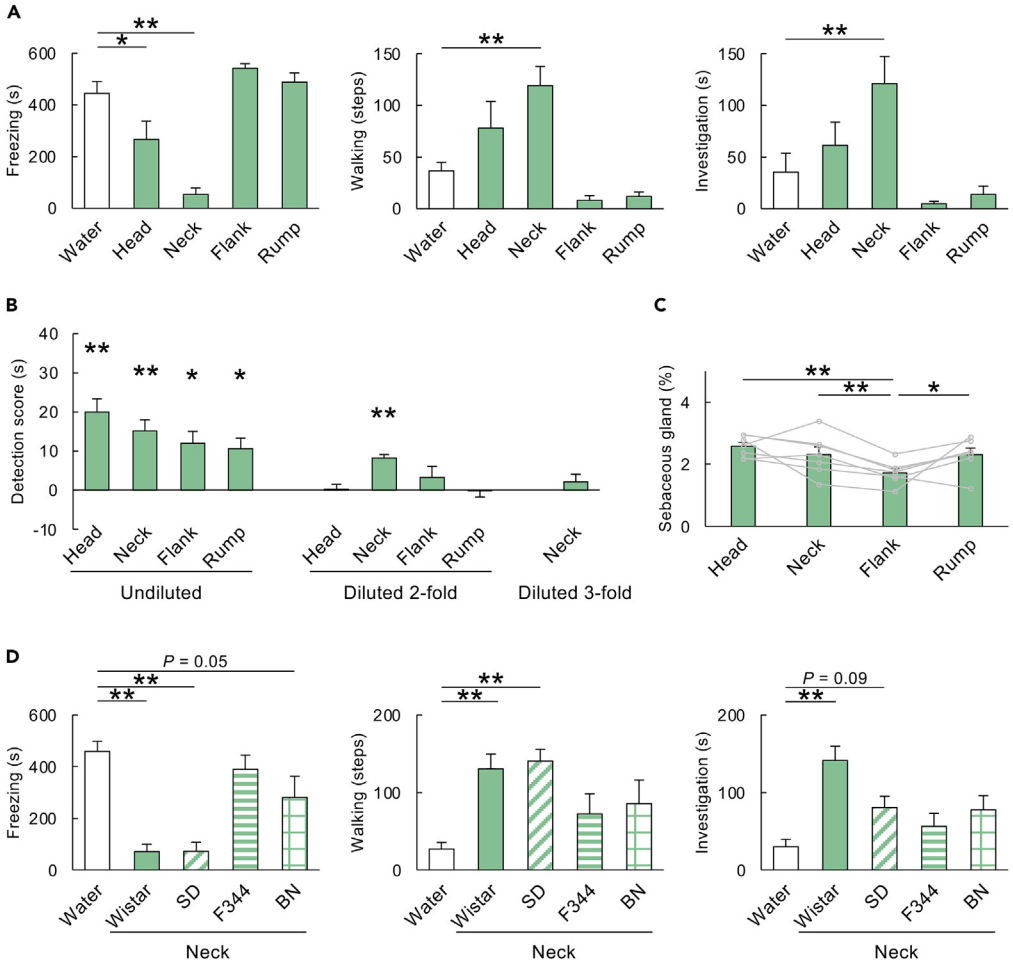


Figure 1. Establishing a method to collect bioactive odors in laboratory rats

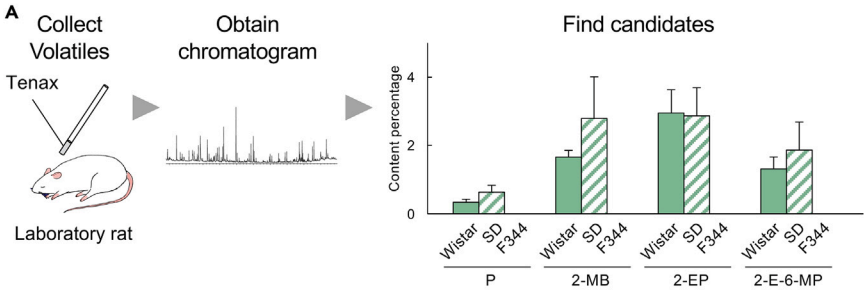
(A) Conditioned fear response of Wistar rats exposed to the aversive CS in the presence of purified water (n = 7) or water carrying the head (n = 7), neck (n = 7), flank (n = 6), or rump (n = 6) odors of other Wistar rats. **p < 0.01, *p < 0.05 by MANOVA ($F(12, 69) = 4.10$, p < 0.01) followed by Dunnett's test. See also Figure S1.

(B) The detection of odor is quantified by a detection score that represents the difference from the third water presentation in the habituation-dishabituation test (n = 6 each). **p < 0.01, *p < 0.05 by paired *t*-test.

(C) The area percentage of sebaceous glands with respect to the area of dermis (n = 6). **p < 0.01, *p < 0.05 by one-way repeated ANOVA ($F(3, 18) = 12.9$, p < 0.01), followed by a paired *t*-test. *p* values corrected using the false discovery rate.

(D) Conditioned fear response of Wistar rats exposed to the aversive CS in the presence of purified water (n = 9) or water carrying the neck odor of other Wistar (n = 9) SD (n = 8) F344 (n = 8), or BN rats (n = 7). **p < 0.01, *p < 0.05 by MANOVA ($F(12, 90) = 6.05$, p < 0.01), followed by Dunnett's test. See also Figure S2. Data are represented as mean + SEM in all panels.

Before continuing the search for pheromone candidates, the obtained chemical profiles were first verified. It is known that rats can categorize unfamiliar rats based on strain.[11] Given that odor plays an important role in the recognition of other rats,[12] specific patterns should be shared among the chemical profiles of rats of the same strain. Consistent with this notion, discriminant analysis accurately separated the obtained profiles into three strains (Figure 2B), even if it seemed difficult to discriminate strains based on the similarity of the content percentage of each chemical (Figure 2C). These results suggest that a sufficiently precise chemical profile of each rat for the further analyses was obtained. The candidates for appeasing pheromone were then searched. By comparing the chemical profiles, it was found that pyrazine (P), 2-MB, 2-ethylpyrazine (2-EP), and 2-ethyl-6-methylpyrazine (2-E−6-MP) were shared by all profiles of Wistar and SD rats, but they were absent in all profiles of F344 rats (Figures 2A and S4). Therefore, these chemicals were considered the candidate chemicals for the appeasing pheromone.



B

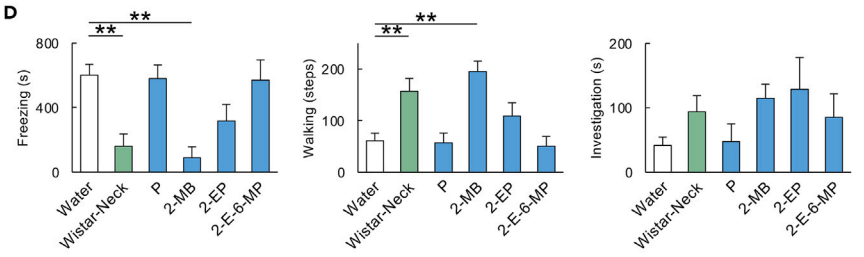
Discriminant analysis

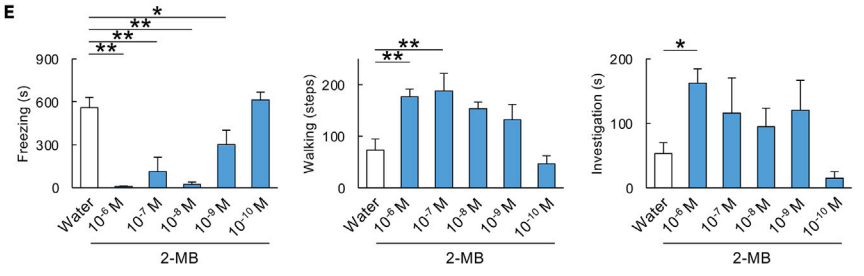
	Percent correct	Classification		
		Wistar	SD	F344
Wistar	100	7	0	0
SD	100	0	7	0
F344	100	0	0	7
Total	100	7	7	7

C

Similarity score

	Wistar	SD	F344
Wistar	66.8	65.4	61.8
SD	-	63.5	60.9
F344	-	-	66.1





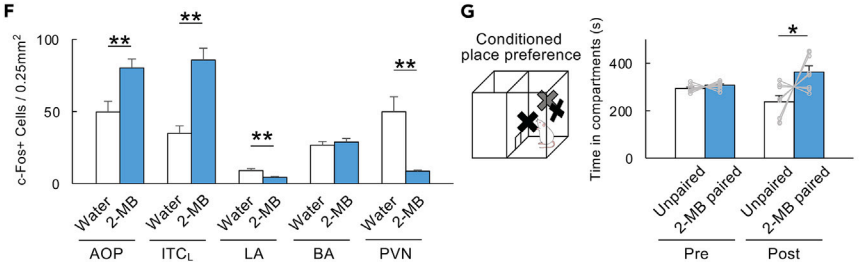


Figure 2. 2-MB functions as an appeasing pheromone that ameliorates fear responses in laboratory rats

(A) A schematic diagram showing the identification of candidate chemicals and the content percentage of P, 2-MB, 2-EP, and 2-E−6-MP in the samples prepared from Wistar, SD, or F344 rats (n = 7 each). See also Figures S3 and S4.

(B) Result of discriminant analysis.

(C) Similarity score of neck odor within and between strains. The similarity score of a pair of rats is defined as the mean value of the percentage of the smaller content percentage with respect to the larger content percentage in 46 chemicals. The similarity score within and between the strains is defined as the mean value of the similarity score in all possible pairs of rats.

(D) Conditioned fear response of Wistar rats exposed to the aversive CS in the presence of purified water (n = 8) or water carrying the neck odor of other Wistar rats (n = 6), 2-MB (n = 7), P (n = 6), 2-EP (n = 6), or 2-E−6-MP (n = 6). **p < 0.01 by MANOVA ($F(15, 86) = 3.62$, $p < 0.01$), followed by Dunnett's test. See also Figure S5.

Figure 2. *Continued*

(E) Conditioned fear response of Wistar rats exposed to the aversive CS in the presence of purified water (n = 7) or 2-MB at a concentration of 1.5×10^{-6} M (n = 8), 10^{-7} M (n = 4), 10^{-8} M (n = 4), 10^{-9} M (n = 6), or 10^{-10} M (n = 5). **p < 0.01, *p < 0.05 by MANOVA ($F(15, 72) = 4.60$, $p < 0.01$), followed by Dunnett's test. See also Figure S6.

(F) Density of c-Fos-immunoreactive cells in the fear-conditioned Wistar rats exposed to the aversive CS in the presence of purified water (n = 7) or 2-MB (n = 8). **p < 0.01 by *t*-test. See also Figure S7.

(G) A schematic diagram showing the conditioned place preference test and time spent by Wistar rats (n = 8) in each compartment in the pre-conditioning (pre) and post-conditioning trial (post). The 2-MP paired compartment had been paired with 2-MB during conditioning. *p < 0.05 by two-way repeated ANOVA (compartment, $F(1, 7) = 6.65$, $p < 0.05$; conditioning, $F(1, 7) = 0$, $p = 1$; compartment × conditioning, $F(1, 7) = 4.63$, $p = 0.0685$), followed by paired *t*-test. Data are represented as mean + SEM in all panels.

The same bioassay was then performed to evaluate the pheromone activity of each candidate chemical. The averaged content percentage represents the ratio of each chemical in the sample. In addition, the absolute amount of these chemicals was estimated at approximately 10^{-6} M by spiking 10 ppm of ethyl nonanoate into additional 10 samples prepared from Wistar rats. Based on these findings, each synthetic molecule was dissolved in purified water at a similar ratio and concentration estimated in the samples prepared from Wistar rats (P, 0.3×10^{-6} M; 2-MB, 1.5×10^{-6} M; 2-EP, 2.6×10^{-6} M; 2-E−6-MP, 1.2×10^{-6} M). It was found that 2-MB, but none of the other chemicals, was as potent as the neck odor of Wistar rats for ameliorating conditioned fear responses, including freezing behavior (Figures 2D and S5). These results suggest that 2-MB alone is capable of ameliorating conditioned fear responses.

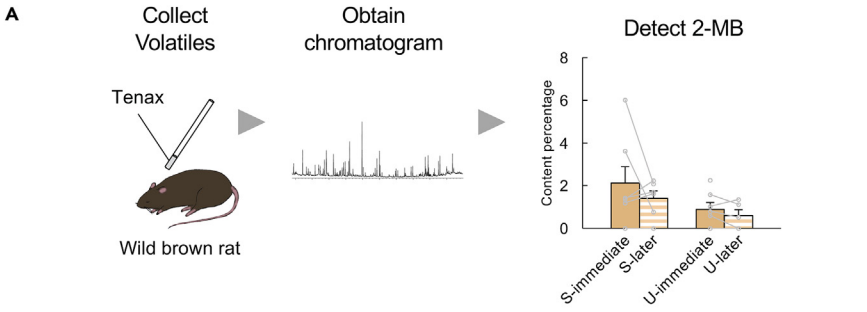
Whether the effects of 2-MB are dose-dependent was then examined. The bioassay showed that freezing was ameliorated even when 2-MB was diluted to 1.5×10^{-9} M (Figures 2E and S6), although the effects on other behavioral measures decreased as 2-MB was diluted (Figure 2E). These results suggest that 2-MB is effective in minute amounts. Furthermore, whether 2-MB and bioactive odor cause the same pattern of brain responses during the amelioration of conditioned fear responses was also examined. When conditioned fear response in Wistar rats is ameliorated by the presence of non-stressed Wistar rats, the volatile odor detected by the main olfactory epithelium activates the posterior complex of the anterior olfactory nucleus (AOP),[13,14] which in turn suppresses the lateral amygdala (LA) and paraventricular nucleus of the hypothalamus (PVN)[13,14] by activating the lateral intercalated cell mass of the amygdala (ITC_L),[15] a cluster of GABAergic neurons. As a result, conditioned fear responses are ameliorated. Based on these findings, the expression of c-Fos protein, a marker of neural activity, was assessed in rats that were exposed to 2-MB at a concentration of 1.5×10^{-6} M during the assay for dose-dependency. It was found that 2-MB increased c-Fos expression in the AOP and ITC_L and decreased c-Fos expression in the LA and PVN (Figures 2F and S7). These results suggest that 2-MB and the bioactive odor share the same neural mechanisms to ameliorate conditioned fear responses.

Whether 2-MB is capable of establishing conditioned place preference in naive rats was then finally assessed (Figure 2G). If 2-MB is an appeasing pheromone and conveys safety, 2-MB is expected to have a rewarding value for naive rats. In the pre-conditioning trial, Wistar rats did not show a preference between the two compartments of the box. During conditioning, one of the two compartments was associated with 2-MB at a concentration of 1.5×10^{-9} M. When the preference was again assessed in the post-conditioning trial without odor presentation, rats showed a preference for the compartment paired with 2-MB (Figure 2G). These results suggest that 2-MB has a reward value for naive rats. On the basis of these findings, we conclude that 2-MB is an appeasing pheromone that ameliorates fear responses in laboratory rats.

An appeasing pheromone in laboratory rats is effective in wild brown rats

Although laboratory rats and wild brown rats are still the same species, *R. norvegicus*, laboratory rats have been isolated from wild brown rats for more than 120 years.[16] Therefore, it is unclear whether 2-MB will function as a pheromone even in wild brown rats. To clarify this point, the effectiveness of 2-MB was explored in wild brown rats.

Whether 2-MB is found in the neck odor of wild brown rats was first evaluated. Seven wild brown rats were caught in each of two different urban centers in Tokyo (places S and U). The neck odor was sampled twice from each rat, once immediately after capture and once after being fed with laboratory chow for one month in the laboratory to reduce the effect of diet (Figure 3A). Accordingly, there were four categories of neck odor samples depending on the trapping place and the collection time point (S-immediate, S-later,



B

Discriminant analysis

	Percent correct	Classification			
		S-immediate	U-immediate	S-later	U-later
S-immediate	100	7	0	0	0
U-immediate	100	0	7	0	0
S-later	100	0	0	6	0
U-later	100	0	0	0	5
Total	100	7	7	6	5

C

Similarity score

	S-immediate	U-immediate	S-later	U-later
S-immediate	66.6	62.9	63.4	61.9
U-immediate	-	68.6	62.6	64.7
S-later	-	-	67.6	64.3
U-later	-	-	-	70.5

D

Proportion of chemical profiles

	Chemicals			
	2-MB	P	2-EP	2-E-6-MP
S-immediate	6/7	0/7	6/7	6/7
S-later	5/6	0/6	5/6	5/6
U-immediate	5/7	0/7	4/7	4/7
U-later	3/5	0/5	3/5	3/5

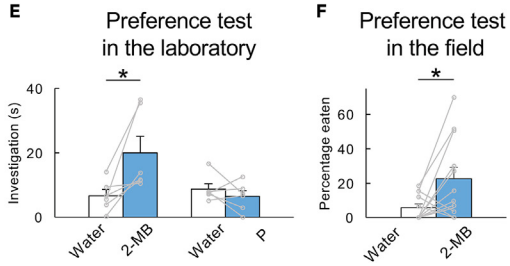


Figure 3. An appeasing pheromone in laboratory rats is effective in wild brown rats

(A) A schematic diagram showing the detection of 2-MB in wild brown rats and the content percentage of 2-MB in the samples in the S-immediate (n = 7), S-later (n = 6), U-immediate (n = 7), and U-later (n = 5) categories. See also Figure S8.

(B) Result of discriminant analysis.

(C) Similarity score of neck odor within and between the categories of wild brown rats. The similarity score of a pair of wild rats is defined as the mean value of the percentage of the smaller content percentage with respect to the larger content percentage in 28 chemicals. The similarity score within and between the categories is defined as the mean value of the similarity score in all possible pairs of wild rats.

(D) Proportion of chemical profiles of wild brown rats in each category that contains 2-MB, P, 2-EP, and 2-E—6-MP.

(E) Duration of investigation of a pair of 2-MB-scented and non-scented trays (n = 6) or a pair of P-scented and non-scented trays (n = 6). *p < 0.05 by paired *t*-test.

(F) Percentage eaten from the 2-MB scented and non-scented trays placed in the field (n = 12). *p < 0.05 by paired *t*-test. See also Figure S10. Data are represented as mean + SEM in all panels.

U-immediate, and U-later). Analysis of the samples in the S-immediate category by GC-MS showed that all chromatograms shared 38 chemicals. Of them, 10 chemicals were most likely environmental contaminants (e.g., benzaldehyde, phenol, etc.). Therefore, the examination focused on 28 chemicals, and the chemical profile of each sample was obtained by calculating the content percentage of each chemical with respect to these 28 chemicals (Figure S8).

Both genetic factors[17] and consumed diet[18] affect body odor. Based on the ecology of wild brown rats in urban cities,[19] rats caught at places S and U most likely belong to genetically different clusters. In addition, the nutrient composition of garbage in the two places and laboratory chow are considered to be different. Consistent with these differences, discriminant analysis accurately separated the obtained profiles into four categories (Figure 3B). Again, it seemed difficult to discriminate the categories based on the similarity in the content percentages of 28 chemicals (Figure 3C). These results indicate that a sufficiently precise chemical profile of each wild brown rat for the further analyses was obtained. The presence of 2-MB in each profile was then searched. It was found that a substantial proportion of profiles in all categories contained

2-MB (Figures 3A and 3D). Additional analyses showed that 2-EP and 2-E−6-MP existed in similar proportions in the profiles, whereas none of the profiles contained P (Figure 3D). Therefore, it is probable that wild brown rats also use 2-MB in their chemical communication.

The effects of 2-MB on wild brown rats were then evaluated. Given that it is practically impossible to measure conditioned fear responses, especially in the field, a preference test between 2-MB-scented and non-scented novel trays was conducted. It is known that most wild brown rats show avoidance of novel objects, i.e., neophobia. Our previous studies suggest that neophobia in wild brown rats is one of the fear responses associated with basolateral complex of the amygdala (BLA) activation.[20] Therefore, it was expected that wild brown rats would show a preference for the 2-MB-scented tray if 2-MB suppresses BLA activity and therefore ameliorates neophobia caused by the novel tray. In the preference test performed in the laboratory, trapped wild brown rats were allowed to explore a pair of 2-MB-scented and non-scented trays or a pair of P-scented and non-scented trays in a familiar experimental room. A preliminary study showed that laboratory rats preferentially investigated the 2-MB-scented tray in this setting (Figure S9). It was found that wild brown rats preferentially investigated the 2-MB-scented tray, but not the P-scented tray (Figure 3E). In the field, the preference test was performed on a poultry farm in summer and in a park in an urban center in winter (Figure S10). Precedent inspections by pest management professionals and rodent control campaigns performed after the preference test confirmed that these testing places were infested exclusively by brown rats. A pair of 2-MB-scented and non-scented trays carrying bait was placed at each test point located throughout the farm or park in a counterbalanced manner for a few hours. When the consumed bait was compared between the two trays at each point, wild brown rats living in the field preferentially consumed bait from the 2-MB-scented tray (Figure 3F). These results suggest that 2-MB ameliorates neophobia in wild brown rats. Taken together, we conclude that 2-MB is an appeasing pheromone in the brown rat (*R. norvegicus*).

DISCUSSION

In the present study, we first established a method to collect the bioactive odor from anesthetized laboratory rats. Subsequent analyses showed that a minute amount of 2-methylbutyric acid (2-MB) was as effective as the bioactive odor. In addition, 2-MB and the bioactive odor produced the same pattern of neural activity when ameliorating conditioned fear responses. Furthermore, when presented to non-stressed rats, 2-MB was found to have a rewarding value. When the effectiveness of 2-MB in wild brown rats was explored, it was found that a substantial proportion of wild brown rats released 2-MB. In addition, 2-MB ameliorated neophobia both in the laboratory and in the field. Taken together, it was concluded that 2-MB functions as an appeasing pheromone that ameliorates fear responses in the brown rat (*R. norvegicus*).

The present study demonstrated the bioactivity of 2-MB in the brown rat. We suggest that 2-MB is a component of body odor spontaneously released from any rat, rather than a specific chemical that is released only from relaxed rats. It is suggested that 2-MB is produced as a byproduct of the catabolism of isoleucine, one of the essential amino acids.[21] Isoleucine is first deaminated by branched-chain amino acid aminotransferase to form 3-methyl-2-oxovaleric acid. Then, 3-methyl-2-oxovaleric acid is oxidatively decarboxylated by the branched-chain α-keto acid dehydrogenase to form 2-methylbutyryl-CoA. Whereas 2-methylbutyryl-CoA is mostly dehydrogenized by short-chain acyl-CoA dehydrogenase to form tiglyl-CoA, some 2-methylbutyryl-CoA is transformed to 2-MB by a thermodynamic spontaneous reaction and subsequent keto-enol tautomerization. In addition, 2-MB can also be produced by gut microbiota and absorbed through the colonic epithelium.[22] Then, 2-MB is metabolized to 2-hydroxy-2-methylbutyric acid, 2-methyl-3-hydroxybutyric acid, 2-ethylhydracrylic acid, ethylmalonic acid, mesaconic acid, and/or 2-methylbutyrylglycine and excreted in urine.[23] Therefore, a certain level of 2-MB is considered to exist throughout the body of brown rats.

Fear responses are considered to be modulated through olfactory communication between same-sex adult individuals as follows. Rats show residual intrinsic responses after suppression by the detected signals.[24] Of the signals, 2-MB induces fundamental amelioration. Self-released 2-MB and/or recognition of the presence of conspecifics through 2-MB may also make a contribution. In addition, the effects of 2-MB are further modified by other signals. For example, the effects are enhanced when 2-MB is released from familiar individuals. The same donor's body odor was found to have stronger effects on the rats that were familiar with the donor.[7] Therefore, it is reasonable to assume the existence of an additional neural mechanism that evaluates familiarity with the donor and enhances the effects of 2-MB depending on the familiarity. In contrast, the effects of 2-MB are reduced when 2-MB is released from distressed rats. Fear responses were less effectively ameliorated by the presence of distressed rats.[25] We have also

demonstrated that distressed rats release the alarm pheromone that evokes anxiety responses by activating the bed nucleus of the stria terminalis (BNST).[26] In addition, the BNST was not involved in the amelioration of fear responses by conspecifics' odor.[14] Therefore, 2-MB and the alarm pheromone are expected to compete with each other. Together with auditory communication,[27] these various forms of olfactory communication are thought to be the basis of the social life of the brown rat.

In contrast, we hypothesize that 2-MB serves as a kairomone in mice. In the literature, an exploratory analysis of a variety of odorants reported that laboratory mice showed strong avoidance of 2-MB at a concentration of 1.7×10^{-6} M or higher.[28] In addition, a subset of olfactory receptors that contribute to the avoidance of 2-MB has been specified.[29] Since 2-MB is one of the aroma compositions of fermented foods and drinks, including cheese[30,31] and wine,[32] the avoidance of 2-MB has been interpreted as the avoidance of spoiled foods. However, the present study demonstrated that a similar concentration of 2-MB ameliorated fear responses in brown rats. It was further demonstrated that 2-MB had a rewarding value for laboratory rats. Given that rats are predators of mice,[33,34] a more probable interpretation of the avoidance behavior observed in mice would be that 2-MB signals the presence of predators, i.e., rats. Whether 2-MB is absent in the body odor of mice should be tested in future studies.

In the present study, discriminant analysis successfully discriminated three strains of laboratory rats, as well as the four categories of wild brown rats. These results suggest the existence of a signature mixture of chemicals in brown rats. A signature mixture is a subset of the molecules in an animal's chemical profile that can be used to recognize an animal as an individual or as a member of a particular social group.[35] The role of the signature mixture in individual recognition has been reported both in social insects[36] and mammals.[37,38] The signature mixture is also used to recognize social group membership for an unfamiliar individual in social insects. For example, ants regard an unfamiliar individual as a member of their own colony if the individual shares a pattern of cuticular hydrocarbons specific to their own colony.[39,40] However, in mammals, the role of the signature mixture in recognition of social group membership is poorly understood. We recently demonstrated that laboratory rats change their social behavior depending on the strain of the unfamiliar rat.[11,41] Therefore, it is highly possible that rats can also recognize the social group of an unfamiliar individual based on its signature mixture.

The present study showed that brown rats release 2-MB strongly from their neck region. This characteristic can contribute to the expression of play behavior in this species. Play behavior is known to be important for the development of social, cognitive, emotional, and sensory motor skills.[42,43] Both in laboratory rats[44] and wild brown rats,[45] play behavior typically starts with one rat soliciting another rat by attempting to make physical contact to the neck region. Play behavior continues when the solicited rat responds to the solicitation and fully rotates to its dorsal surface. Indeed, local anesthesia to the neck region reduced the reactivity to the solicitation.[46] In addition, experimenters can elicit play behavior by tickling the neck region of laboratory rats.[47] Therefore, it is possible that some play solicitations are attempts to investigate the source of 2-MB. Further research is needed to clarify the adaptive significance of appeasing pheromones in this species.

In conclusion, an appeasing pheromone that ameliorates fear responses in brown rats (*R. norvegicus*) was identified. The present findings might contribute both to furthering the advancement of science and improving public health. It is reasonable to assume that the appeasing pheromone is involved in social skills of laboratory rats, e.g., helping behavior, consolation behavior, and social buffering. Therefore, a better understanding of pheromonal communication might enable us to analyze social skills more effectively. In addition, the identification of appeasing pheromone allows us to develop novel methods for pest control campaigns. Because wild brown rats show neophobia, culling programs using traps and rodenticides, i.e., novel objects, cannot control brown rats effectively. In addition, as indicated by the fact that the use of inhumane glue traps will be banned in the United Kingdom from April 2024, culling programs are being required to use more humane methods. Therefore, the appeasing pheromone might help us to perform more effective pest management campaigns with low animal welfare impacts.

Limitations of the study

Because we primarily focused on assessing whether 2-MB functions as a pheromone both in laboratory rats and wild brown rats, we did not answer the following interesting questions. First, it is unclear why we did not detect 2-MB in F344 rats. It is possible that the activities of isoleucine catabolism and/or 2-MB metabolism are different in F344 rats. Second, because 2-MB is chiral, it is unclear which or both enantiomers function as

an appeasing pheromone. However, the present study demonstrated that 2-MB functions as an appeasing pheromone even in a racemic mixture. Third, it is unclear why we did not detect isovaleric acid and isobutyric acid in any rats. These chemicals are structurally similar to 2-MB and are theoretically produced as a byproduct of leucine and valine catabolism, respectively. Given that leucine and valine are also essential amino acids, their catabolism should occur in all rats. However, to the best of our knowledge, none of the studies, including ours, has detected these chemicals in the volatile odor of mammals. Fourth, although the presence of a non-stressed rat ameliorates fear responses through the main olfactory system,[6] it remains to be explicitly demonstrated whether the effects of 2-MB are mediated by the main olfactory system. Further research is needed to answer these questions.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107081.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.K.; Data curation, Y.K.; Formal analysis, Y.K., M.F.; Funding acquisition, Y.K., Y.T.; Investigation, Y.K., S.T., M.O., E.K., D.M.; Methodology, Y.K., S.T., M.F.; Project administration, Y.K.; Resources, Y.K., M.F., S.N., T.T.; Supervision, Y.K.; Validation, Y.K.; Writing-original draft, Y.K., M.F.; Writing-review & editing, Y.K., M.F., Y.T.

DECLARATION OF INTERESTS

The authors (Y.K., S.T., and S.N.) have a patent application that is based on this study.

INCLUSION AND DIVERSITY

We support inclusive, diverse and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal anti-c-Fos	Cell Signaling Technology	Cat#2250; RRID: AB_2247211
Goat anti-Rabbit IgG, Biotinylated	Vector Laboratories	Cat#BA-1000; RRID:AB_2313606
Mouse monoclonal anti-GAD67	Millipore	Cat#MAB5406; RRID:AB_2278725
Horse anti-Mouse IgG, Biotinylated	Vector Laboratories	Cat#BA-2000; RRID:AB_2313581
Chemicals, peptides, and recombinant proteins		
DL-2-Methylbutyric acid	Tokyo Chemical Industry	Cat#M0181; CAS: 116-53-0
2-Ethylpyrazine	Tokyo Chemical Industry	Cat#E0334; CAS: 13925-00-3
Pyrazine	Tokyo Chemical Industry	Cat#P0544; CAS: 290-37-9
2-Ethyl-6-methylpyrazine	aablocks	Cat#AA003H4L; CAS: 13925-03-6
Ethyl nonanoate	Tokyo Chemical Industry	Cat#N0289; CAS: 123-29-5
Experimental models: Organisms/strains		
Rat: Wistar: Crlj:WI	The Jackson Laboratory	N/A
Rat: Sprague-Dawley: Crl:CD(SD)	The Jackson Laboratory	N/A
Rat: Fischer344: F344/DuCrlCrlj	The Jackson Laboratory	N/A
Rat: Brown Norway: BN/CrlCrlj	The Jackson Laboratory	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yasushi Kiyokawa (akiyo@mail.ecc.u-tokyo.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

All experiments were approved by the Animal Care and Use Committee of the Faculty of Agriculture of The University of Tokyo or the local authorities in Germany (Landesverwaltungsamt Sachsen-Anhalt; Az.42502-2-1587 UniMD), based on guidelines adapted from the consensus recommendations on effective Institutional Animal Care and Use Committees by the Scientists Center for Animal Welfare.

To perform bioassays, perform the habituation-dishabituation test, analyze the sebaceous glands, and obtain chemical profiles, experimentally naive male rats of different strains were purchased from Charles River Laboratories Japan (Kanagawa, Japan): Wistar (aged 7.5 weeks), Sprague-Dawley (SD; aged 8 weeks), Fischer344 (F344; aged 9 weeks), and Brown Norway (BN; aged 9 weeks). To ensure similar body size among strains, rats of different ages were ordered depending on the strain.[10] On arrival, rats of the same strain

were housed with 2–3 animals in a temperature (24 ± 1°C)- and humidity (45 ± 10%)-controlled room on a 12-h light-dark cycle (lights on from 8:00 to 20:00). Food and water were available *ad libitum*. A day after arrival, all rats subjected to the bioassay were housed individually and handled for 5 min per day for 3 days before the fear conditioning day.

To perform the conditioned place preference test, experimentally naive male Wistar rats (aged 8 weeks) were taken from the institute's breeding colony (University of Magdeburg). The animals were housed with 4–6 animals in a temperature (21 ± 1°C)- and humidity (55 ± 10%)-controlled room on a 12-h light-dark cycle (lights on from 6:00 to 18:00) with food and water available *ad libitum*. These subjects were handled 1–2 times before the test.

To obtain chemical profiles, male wild brown rats (204.8 ± 20.4 g) were trapped within 200 m of two different urban centers in Tokyo (places S and U), which are 10 km apart. Immediately after capture, they were transferred to the laboratory at Ikari Shodoku Corporation. On arrival, the wild brown rats were weighed and soaked in an etofenprox solution 3 times and housed individually in wire mesh cages (23.5 × 40 × 16.5 cm) in a room with an ambient temperature of 20 ± 5°C and a 12-h light/dark cycle (lights were switched on at 8:00). Food (CE-2, Clea Japan, Tokyo, Japan) and water were available *ad libitum*.

To perform the preference test in the laboratory, male wild brown rats (146.6 ± 12.5 g) were trapped at a poultry farm in Nagano Prefecture approximately one month before the test. Immediately after capture, they were transferred to a laboratory at Daimaru Compound Chemical. On arrival, the wild brown rats were housed with 2–4 rats in standard cages (25.5 × 40.5 × 20 cm) in a room with an ambient temperature of 20 ± 10°C and a 10-h light/14-h dark cycle (lights were switched on at 8:00). Food (MF, Oriental Yeast, Tokyo, Japan) and water were available *ad libitum*. Starting approximately one week before the test, wild brown rats were housed individually, and a plastic box (12.5 × 22 × 8.8 cm) with a small round hole (diameter 6.5 cm) in the center of one of two smaller walls was placed in each home cage at this time.

To perform a preliminary preference test in the laboratory, experimentally naive male Wistar rats (aged 7.5 weeks) were purchased from Charles River Laboratories Japan. A day after arrival, all rats were housed individually and handled for 5 min per day for 3 days before the preliminary study. No plastic box was placed in each home cage.

METHOD DETAILS

Preparation of water carrying volatile odor samples

All procedures were performed between 13:00 and 17:00, as described in our previous studies.[9] Briefly, purified water (5 mL) was first sprayed on the ceiling of an acrylic box (20 × 20 × 10 cm). Each donor rat was anesthetized with sodium pentobarbital, and intradermal needles (27 G) for electrical stimulation were placed in one region: head, neck, flank, or rump. The donor was then placed in the box for 15 min. During this period, the donor rat received 15 electrical stimuli (10 V for 1 s) at 1-min intervals through the needle. It was confirmed that needle pricking and electrical stimulation did not evoke any bleeding or apparent damage to the skin. After being stimulated in this manner, the donor was removed, and the water droplets on the ceiling were collected in a glass tube using a glass bar and Pasteur pipette, and 3 mL of these samples were placed in a glass vial and stored in a freezer at −20°C overnight until use. As a control sample, water droplets were collected from a box in which no rat had been placed. The donors were not used repeatedly. The box was washed in hot water with a cleanser and wiped with a paper towel before each use.

Candidate chemical pheromone candidates were purchased as follows: pyrazine (P), 2-methylbutyric acid (2-MB), and 2-ethylpyrazine (2-EP) from Tokyo Chemical Industry, Tokyo, Japan, and 2-ethyl-6-methylpyrazine (2-E−6-MP) from aablocks, CA, USA. Each candidate chemical was dissolved in purified water, and purified water was used as a control sample.

Bioassay for the amelioration of fear responses

Auditory fear conditioning was performed in an illuminated room between 9:00 and 13:00, as described in our previous studies.[48] The subject was placed in an acrylic conditioning box (28 × 20 × 27 cm) for 20 min, where it received seven repetitions of a 3-s tone (CS, 8 kHz, 70 dB) that terminated concurrently with a foot shock (0.5 s, 0.3 mA). The intertrial interval was randomly varied from 30 to 180 s. The subjects were returned to their home cages after fear conditioning.

A bioassay was performed in a dark room illuminated by dim red light 24 h after the fear conditioning. Three milliliters of water to be assessed were dropped on four sheets of 5 × 5 cm^2 filter paper. These filter papers were placed on both sides of the wall of an acrylic test box with a perforated ceiling (25 × 25 × 25 cm). A subject was then placed in the test box. After an initial 5-min acclimation period, a 3-s CS tone was presented five times at 1-min intervals during the first 5 min of the 10-min (water samples prepared from anesthetized rats) or 15-min (candidate chemicals) experimental period. The behaviors of the subjects during the acclimation and experimental periods were recorded with a video camera (HDR-HC9, Sony, Tokyo, Japan) and an HDD-BD recorder (DMR-BW770; Panasonic, Osaka, Japan).

An experimenter who was blinded to the experimental conditions recorded behavioral responses using a Microsoft Excel-based Visual Basic program that recorded the duration and number of key presses. The duration of freezing behavior (an immobile posture with cessation of skeletal and vibrissae movements, except for those related to respiration) and investigation behavior (making direct contact with the filter paper, regardless of its mode of attachment on the wall, including chewing or the use of vibrissae), and frequency of walking behavior (number of steps taken with the hind paws) of the subjects were recorded regardless of the presence or absence of the CS.

Habituation–dishabituation test

The habituation–dishabituation test was conducted in a colony room between 9:00 and 13:00, as described in our previous studies.[49] After removing the water bottle on the stainless-steel cage top, each subject was transported in its home cage to another shelf in the colony room. After a 30-s acclimation period, purified water was presented by pipetting 50 μL onto one-half of a filter paper (5 × 5 cm) attached to the edge of the ceiling, such that the rat was unable to make physical contact with the filter paper and only volatile odors from the stimulus were available at body level. After three consecutive 2-min presentations of purified water, the sample to be assessed was presented for 2 min, at 30-s intervals. The behavior of the subject was video-recorded (DCR-DVD403; Sony) for later analysis.

A researcher blinded to the experimental conditions recorded the investigation time (time spent sniffing toward the stimulus and poking the nose into the ceiling) of each stimulus using a Visual Basic program, as previously described. A detection score was calculated by subtracting the investigation time of the third purified water from the investigation time of the sample.

Analyses of sebaceous glands

The deeply anesthetized Wistar rats were perfused intracardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1-M phosphate buffer. A piece of skin (2 × 2 cm) was sampled from the head, neck, flank, and rump region, and immersed in the same fixative. The paraffin-embedded skin was cut into 2 to 3-μm-thick sections and stained with hematoxylin and eosin.

A researcher blinded to the experimental conditions measured the area of dermis, the area of sebaceous glands, and the number of sebaceous glands in the dermis of an approximately 1-cm length of skin sample using ImageJ software (version 1.51). The area percentage of sebaceous glands with respect to the area of dermis was calculated.

Analyses of neck odor

Collection of neck odor was performed in an acrylic box (25 × 25 × 25 cm) filled with purified air (G3: Nippon Megacare, Tokyo, Japan). The neck region of an anesthetized rat was electrically stimulated for 1 h using the same method as described above. During electrical stimulation, the air around the neck region was sucked into an inlet liner (5181-3316, Agilent, CA, USA) packed with 0.25 g of adsorbent Tenax (TENAX-TA 30/60 mesh, Buchem B.V., Apeldoorn, Netherlands), using a suction pump (50 mL/min; MP-2N, Sibata Scientific Technology, Tokyo, Japan). The inlet liner was then transported to, and subsequently desorbed in, the thermal desorption system (HandyTD TD265, GL Sciences, Tokyo, Japan). The thermal desorption system was programmed from 40°C to 230°C (held for 2 min) at 45°C/s with 50 mL/min desorption flow. Desorbed compounds were kept at −196°C on a fused silica capillary tube in the handmade cooled injection system inlet. The cooled injection system was programmed from −196°C to 180°C (held for 10 min) at 30°C/min to inject trapped compounds into the analytical column.

GC-MS analyses were performed using an Agilent 7890B GC combined with a 5977A mass selective detector equipped with an InertCap1 capillary column (I. D. = 0.25 mm, 60 m length, df = 0.25 μm; GL Sciences). The oven temperature was maintained at 40°C for 5 min and then increased to 220 °C at a rate of 3°C/min, with a constant carrier helium gas flow of 1.0 mL/min. Mass spectra in the electron impact mode were recorded at an ionization energy of 70 eV. Each peak in the chromatogram was specified using an Agilent MSD productivity ChemStation GC and NIST Library.

Of the 63 chemicals identified in the neck odor released from laboratory rats, the evaluation focused on 46 chemicals that appeared to not be contaminants from the environment. Similarly, the evaluation focused on 28 of 38 chemicals in the neck odor released from wild brown rats because they appeared to not be environmental contaminants. The area under the peak was measured for each chemical, and its percentage with respect to the area of 46 and 28 chemicals, respectively, was expressed as a content percentage.

To estimate the absolute concentration of four candidates, 10 additional adsorbents that had collected the neck odor of Wistar rats were prepared, as described above. Then, the inlet liners containing adsorbents were attached to the Agilent GC, and 10 μL of 0.0001% ethyl nonanoate (10 ppm, Tokyo Chemical Industry) diluted in pentane were injected with a constant carrier nitrogen gas flow of 50 mL/min as an internal standard. Ethyl nonanoate was chosen because it did not overlap any peaks in the sample. These adsorbents were subsequently analyzed as described above.

The similarity score of a pair of donors was defined as the mean value of the percentage of the smaller content percentage with respect to the larger content percentage in all chemicals (i.e., 46 chemicals in laboratory rats and 28 chemicals in wild brown rats). The similarity score within and between the strains or groups was defined as the mean value of the similarity score in all possible pairs of donors.

For the discriminant analysis, the top 20 of 46 chemicals in laboratory rats and the top 20 of 28 chemicals in wild brown rats were selected based on the number of rats that released the target chemical such that the content accounted for more than 1% of the total content.

Analyses of Fos expression

After assessment of the dose-dependent effects of 2-MB, the subjects tested in the presence of 2-MB at a concentration of 1.5×10^{-6} M or purified water were returned to their home cages and kept in the colony room. Then, each subject was deeply anesthetized with sodium pentobarbital 45 min after the bioassay, i.e., 60 min after the first tone delivery, and perfused intracardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1-M phosphate buffer. The brain was sampled, immersed overnight in the same fixative, and then placed in 30% sucrose/phosphate buffer for cryoprotection. The avidin-biotin-peroxidase method was used to detect immunoreactive cells, as described previously.[50] Briefly, six serial 30-μm-thick sections containing the AOP (bregma 3.24 mm), PVN (bregma −1.80 mm), or LA, BA, and ITC_L (bregma −3.00 mm) were collected. It was not possible to obtain sections containing the AOP of 1 animal in the Water group due to technical problems. The sections were first stained for Fos protein. After incubation in 0.3% H_2O_2, the free-floating sections were incubated in citrate buffer (B442, LSI Medience Corporation, Tokyo, Japan) at 60°C for 2 h. The sections were then incubated with anti-c-Fos antibody (1:7500, 2250, Cell Signaling Technology, Danvers, MA, USA) for 65 h at 4°C, followed by incubation with biotinylated anti-rabbit secondary antibody (BA-1000, Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature. The sections were then incubated in VECTASTAIN Elite ABC reagent (PK-6100, Vector Laboratories) and developed using a diaminobenzidine solution with nickel intensification. Sections containing the PVN and ITC_L were further stained for glutamate decarboxylase (GAD) 67 protein to locate the ITC_L. Sections were incubated with anti-GAD67 antibody (1:4000, MAB5406, Merck Millipore) overnight at 4°C, followed by incubation with biotinylated anti-mouse secondary antibody (BA-2000, Vector Laboratories) for 2 h at room temperature. The sections were then incubated with VECTASTAIN Elite ABC reagent and developed using a diaminobenzidine solution without nickel intensification.

Six sections of each region were captured using a microscope equipped with a digital camera (DP30BW, Olympus, Tokyo, Japan). An experimenter who was blinded to the experimental conditions randomly selected three sections in the left hemisphere and three sections in the right hemisphere for each nucleus. The number of Fos-immunoreactive cells was counted within a 0.5 mm × 0.5 mm square of each nucleus using ImageJ software (version 1.51). When the designated area was smaller than the boundaries of the

square, only the cells within the nucleus were counted. Because the ITC_L is a cluster of GABAergic neurons located lateral to the BLA and much smaller than a 0.5 mm × 0.5 mm square, the GAD67-immunoreactive areas located lateral to the BLA were measured as the areas of the ITC_L. The number of Fos-immunoreactive cells within the area was then counted. In all regions, the density (immunoreactive cells/0.25 mm^2) was calculated.

Conditioned place preference test

The test was performed at the University of Magdeburg. Trials and conditioning were performed in a box (49.5 × 49.5 × 41.5 cm) that was separated into two compartments with similar sizes and similar illumination (10–15 lux). One compartment had dark walls, and the other compartment had three white walls with a single big black cross on each of them. The wall between the compartments was dark and had an opening (8 × 6 cm) in the middle that was open during the trials, but closed during conditioning. The box was equipped with infrared detector frames (sensor distance: 16 mm) to allow tracking the rats' positions and movements (TSE Systems, Bad Homburg, Germany).

In the pre-conditioning trial, the subjects were allowed to freely explore two compartments without odor presentation for 10 min. Seven-day conditioning was performed from 1 day after the pre-conditioning trial. The subjects were placed in the 2-MB paired compartment for 10 min twice daily and in the unpaired compartment for 10 min twice daily. Five sheets of 5 × 5 cm filter paper carrying 3.5 mL of 2-MB at a concentration of 1.5×10^{-9} M were placed on three walls of the 2-MP paired compartment. Purified water was used for the unpaired compartment. The allocation of 2-MB paired and unpaired compartments was balanced between the subjects. One day after the end of conditioning, the post-conditioning trial was performed, which was identical to the pre-conditioning trial. The time spent in each compartment was measured automatically.

Preference test in the laboratory

The preference test was performed in a dark trapezoid room (210 × 304 × 227 × 306 cm) illuminated by dim red light between 9:00 and 1500. Before the preference test, the subjects had been acclimatized to the experimental room. The plastic box with the subject was gently placed at the center of the experimental room and kept there for 10 min. If the subjects did not come out from the plastic box and explore the entire area of the experimental room, an additional 10 to 20 min was allowed. This procedure was repeated 3 to 4 times during 3–4 days before the test so that all subjects readily came out from the plastic box.

On the test day, silica gel beads (4-mm diameter) were soaked either in a 2-MB (1.5×10^{-2} M), P (0.3×10^{-2} M), or purified water. Then, 7 g of each type of beads were air-dried for approximately 2 h and placed on a red bait tray (11.5 × 11.5 cm). Immediately before the preference test, a pair of trays carrying 2-MB and water beads or carrying P and water beads were placed at two corners of the experimental rooms. Then, the plastic box with the subjects was placed at the center of the experimental room. The behaviors of the subjects during the subsequent 10-min period were recorded with a night vision camera (WTW-KN529-V2, Wireless Tsukamoto, Mie, Japan) and an HDD-BD recorder (BDZ-ZT1700; Sony).

A preliminary study was performed as described above with the exception that the Wistar subjects had not been acclimatized to the experimental room. In addition, on the test day, the subjects alone were gently placed at the center of the experimental room.

A researcher blinded to the experimental conditions recorded the investigation time (time spent sniffing toward the beads or tray) for each tray using Visual Basic software, as previously described.

Preference test in the field

The preference test was performed on a poultry farm in Nagano Prefecture from 26 to 27 August 2020 and in a park in an urban center of Tokyo from 5 to 6 November 2020. Precedent inspections by pest management professionals and rodent control campaigns performed after the preference test confirmed that these testing places were infested exclusively by brown rats. On the poultry farm, a pair of white dishes (15-cm diameter) carrying 100 g of bait and 30 g of either 2-MB or water beads were placed in a counterbalanced manner at 17 test points throughout 10 b around 7 p.m. (one to three test points in each barn). In the park, a pair of red bait trays (11.5 × 11.5 cm) carrying 20 g of bait and 7 g of either 2-MB or water beads were placed around midnight in a counterbalanced manner at five test points close to a garbage station

each. In both places, the amount of bait consumed was measured around 2 h later. When no bait was found to have been eaten at this check, the dishes/trays were returned to the same places and re-surveyed a few hours later. Eight test points in the poultry farm were excluded, as well as two test points in the park, from the analyses because no bait was eaten from both types of dishes/trays even at the second observation at these points. The amount of bait consumed per point was calculated and expressed as a percentage with respect to the initial amount of bait.

QUANTIFICATION AND STATISTICAL ANALYSIS

All of the statistical details of experiments can be found in the figure legends, including the statistical tests used, exact value of *n*, what *n* represents, and dispersion and precision measures (mean ± standard error of the mean). The significance criterion for all statistical tests was set at $p < 0.05$. Although no statistical methods were used for sample size estimation, sample sizes were consistent with those of our previous studies.[4,5,7–11,13–15,51] All animals were allocated to each group upon arrival, even if no statistical methods were used to determine strategies for randomization.

Statistical analyses were performed using JMP Pro 16 (JMP Japan, Tokyo, Japan). In the bioassays, the total duration of freezing and investigation and total frequency of walking during the experimental period were analyzed by one-way multiple analysis of variance (MANOVA) followed by Dunnett's post hoc test. In the habituation-dishabituation test, the detection score was analyzed by paired *t*-test. To assess the prevalence of sebaceous glands, the area percentage of sebaceous glands with respect to the area of dermis was analyzed with one-way repeated ANOVA. Post hoc pairwise comparisons were made by paired *t*-test. Significance was adjusted for multiple comparisons using the Benjamini and Hochberg correction (i.e., false discovery rate).[52] To analyze Fos expression, the density of c-Fos-immunoreactive cells in a 0.25 mm^2 square was analyzed by the *t*-test. In the conditioned place preference test, the time spent in each compartment was analyzed with two-way repeated ANOVA. A planned comparison was conducted using the paired *t*-test. In the preference test in the laboratory, the difference in the investigation time between the trays was analyzed with a paired *t*-test. In the preference test in the field, differences in the percentage of bait consumed were analyzed by a paired *t*-test.