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Aversion- *vs* fear-inducing properties of 2,4,5-trimethyl-3-thiazoline, a component of fox odor, in comparison with those of butyric acid

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SUMMARY

2,4,5-trimethyl-3-thiazoline (TMT), a component of fox feces, is a widely used odorant to induce innate fear behavior in rats and mice. However, based on the slight acrid smell it was argued that the observed behavioral effects are a result of the aversive and not of the fear-inducing properties of TMT. In the present study, we tried to directly compare the aversive and fear-inducing properties of TMT with those of the aversive control odor butyric acid. We first identified concentrations of butyric acid and TMT that induce similar amounts of avoidance behavior in rats, indicating that these concentrations have similar aversive properties. In a second experiment, these two concentrations were then tested for their ability to induce freezing, a species-specific defensive response. Only TMT but not butyric acid induced freezing in the rats. This supports the hypothesis that TMT indeed has specific fear-inducing properties and that the observed behavioral effects could not simply be reduced to the aversive properties of TMT.

Key words: predator odor, fear, avoidance, freezing.

INTRODUCTION

In recent years, an increasing number of studies used the synthetically derived component of fox feces 2,4,5-trimethyl-3-thiazoline (TMT) as a fear-inducing stimulus (reviewed by Fendt et al., 2005). The effect TMT has on the behavior of rats is similar to that caused by exposure to the odor of their natural predators such as a cat or a ferret [summarized by Fendt et al. and Fendt and Endres (Fendt et al., 2005; Fendt and Endres, 2008)]. Exposure to both TMT and natural predator odors induce freezing behavior in rats and mice as well as other species-specific defensive reactions such as defensive burying, risk assessment and a corticosterone release (Endres et al., 2005; File et al., 1993; Morrow et al., 2002; Morrow et al., 2000; Zangrossi and File, 1992) (e.g. Blanchard et al., 2003; Hebb et al., 2004; Holmes and Galea, 2002; Masini et al., 2005; Takahashi et al., 2005). Unlike the odors of natural predators, TMT is a single molecule and not a combination of different elements. This means the advantage of TMT is that it can be used in fixed and comparable concentrations and that its properties are not affected by further factors that may influence the fear-inducing abilities of the odor, e.g. the diet of the odor donator (Berton et al., 1998). At a first glance, TMT seems to be a particularly good tool for the research on the innate fear in rodents. However, in most studies TMT was used in substantially high concentrations to constitute strong behavioral effects. Furthermore, based on the intense and acrid smell of TMT, it has been argued that rather than a fear-inducing stimulus, TMT is more a noxious stimulus (Blanchard et al., 2003; McGregor et al., 2002). Therefore, the question arises whether observed behavioral and endocrinal changes could be attributed to the aversive or repugnant properties of TMT rather than to its fear-inducing properties.

The aim of the present study is to test whether the aversive or repugnant properties of an odor are also responsible for the apparent fear-inducing effects. Therefore, we compared the properties of TMT with that of butyric acid; another synthetically derived aversive or repugnant odor stimulus, which is frequently used as a control odor in TMT experiments (Endres et al., 2005; Hebb et al., 2004; Morrow et al., 2000; Wallace and Rosen, 2000). To identify their aversive properties, we used an open-field arena to test avoidance behavior of rats exposed to the two odors. After identifying the concentrations of the two odors, which induced the same amount of avoidance behavior, we exposed rats to these odor concentrations in order to measure fear behavior (freezing) during odor exposure. If only TMT but not butyric acid instigate fear behavior (despite the equal amounts of avoidance behavior induced by these particular concentrations), the fear-inducing properties of TMT cannot simply be reduced to its aversive properties.

MATERIALS AND METHODS

We used 40 three-month-old Sprague-Dawley (*Rattus norvegicus* Berkenhout 1769) rats, which were housed in groups of three–four animals and had free access to food and water. There was a 12 h light cycle (light phase starting at 07:30). All behavioral tests were performed during the light phase. All experiments were carried out in accordance with the ethical guidelines for the use of animals in experiments and were approved by the local animal care council (Regierungspräsidium Tübingen, ZP 4/02).

Avoidance behavior

Apparatus

We used a cubic ActiMot open field arena (TSE Systems, Bad Homburg, Germany) with a side length of 92 cm. The location of the rats was determined by a frame with infrared light rays (distance: 2.5 cm), which enabled us to automatically measure the time the rat

spent in each corner of the arena. The corner area was defined as a square of 23 cm side length.

Behavioral testing

Before the experiment started, the rats were habituated to the arena for 10 min on two consecutive days. For the next three days, a piece of filter paper ($2\times2\,cm^2$) was placed into every corner of the arena. One filter paper contained 5 µl of the respective odorant. The corner, as well as the presentation order, was pseudorandomized with each test lasting 10 min. For the odorants we used butyric acid (Merck-Schuchardt, Hohenbrunn, Germany) and TMT [PheroTec, Delta, Canada; diluted with diethylphthalate (DEP; Merck-Schuchardt, Hohenbrunn, Germany)] while pure DEP acted as the control. A group of 10 rats was tested for each odor in pseudorandomized order.

Freezing behavior

To explore the fear-inducing properties of the odor concentrations identified in the avoidance test above, four identical odor exposure boxes made of gray PVC ($30\times30\times30\,cm^3$) were used. The front door was made of clear Plexiglas to enable videotaping of the rats while the backside of the box was connected to an exhaust system. First, we familiarized the rats (three groups of 10 new animals) to the exposure boxes by placing them inside for 5 mins for two consecutive days. On the third day, we added 5 µl of butyric acid, 5 µl 10^{-1} % vol. TMT or 5 µl DEP to a piece of filter paper and placed it directly into the box. Each rat was exposed to only one of the different odors for five minutes each. The video recordings were later analyzed for freezing behavior by an observer who was not aware of the respective animal's test condition. Freezing was defined by a crouched posture of the rat and an absence of all body movements, except for those that are necessary for breathing (Blanchard and Blanchard, 1969).

Statistical analysis

To identify avoidance behavior, the mean time an individual rat spent in the three neutral corners of the arena was compared with the time this rat spent in the odor corner. We then performed an analysis of variance (ANOVA; software: Systat 11, Systat Software, San Jose, CA, USA) with the within-subject factors odor and corner (neutral *vs* odor corner). To analyze freezing behavior, we used an ANOVA with the between-subject factor odor. Pairwise comparisons were made by *post-hoc* Tukey tests. A P-value<0.05 was considered as the statistical criterion.

RESULTS

Avoidance behavior

In a pilot study, we tested different concentrations of TMT and butyric acid for their ability to induce avoidance behavior. In detail, we observed that 5 µl of butyric acid in concentrations of 10^{-1}, 10^{-2} and 10^{-3} % vol. were not able to induce avoidance behavior, i.e. the animals did not spend less time in the corner with butyric acid than in the corner without odor (t-values <0.62, P-values >0.58). Undiluted butyric acid (5 µl, 54.7 µmol) was the lowest concentration to induce moderate avoidance behavior (i.e. the odor corner was significantly but not totally avoided by the animals). For TMT, we observed a strong avoidance (i.e. the animals spent nearly no time in this corner) of the odor corner when 5 µl of undiluted TMT (38.7 µmol) was used. A TMT concentration of 10^{-1} % vol. (3.87 µmol) induced moderate avoidance whereas a concentration of 10^{-2} % vol. (387 nmol) led to a very weak avoidance of the odor corner. Therefore, we decided to compare 5 µl 10^{-1} % vol. TMT (3.87 µmol) with 5 µl sheer butyric acid (54.7 µmol) in the present study because both concentrations seemed similarly effective in inducing moderate but reliable avoidance responses. The solvent DEP served as a control odor.

Fig. 1A depicts the percentage of the mean time the rats spent in the neutral corners compared with the odor corner of the open field. An overall ANOVA with corner and odor as within-subject factors revealed no general effect of the odor ($F_{2,54}$=1.69; P=0.20) but significant effects of the corner ($F_{1,54}$=8.21; P=0.006) as well as (most importantly) a significant interaction between the factors odor and corner ($F_{2,54}$=3.81; P=0.03) were revealed. *Post-hoc* pairwise comparisons showed that the rats spent significantly less time in the corner with TMT and butyric acid samples (t-values >2.65; P-values <0.01) whereas the solvent DEP had no significant effect on the time the rats spent in the respective corner (t=0.6; P=0.72). There was no difference between butyric acid and TMT (t=0.32; P=0.75).

The basal activity of the animals was not influenced by the presentation of the different odors: activity time ($F_{2,27}$=0.50; P=0.61), distance traveled ($F_{2,27}$=0.31; P=0.73), time spent immobile ($F_{2,27}$=0.50; P=0.61) and time spent in the center of the arena ($F_{2,27}$=0.52; P=0.60).

Freezing

In Fig. 1B, the percentage of time spent freezing during exposure to 5 µl butyric acid, 10^{-1} % vol. TMT or DEP is shown. An overall ANOVA with odor as a between-subject factor revealed a significant effect of the odor on freezing behavior ($F_{2,27}$=38.0; P<0.0001). *Post-hoc* pairwise comparisons showed that TMT induced significantly more freezing behavior than either butyric acid or DEP (P-values <0.001) whereas there was no difference between butyric acid and DEP (P=0.93). In addition, one-sided t-tests revealed that the amount of freezing that was induced by butyric acid and DEP did not differ significantly from 0 (t-values <2.21, P-values >0.05), indicating that these two odors did not induce freezing behavior.

In Fig. 1C, the mean time spent in the odor corners (avoidance experiment) and spent freezing (freezing experiment) are depicted in an X–Y graph. The gray area shows the mean ± s.e.m. time spent in the butyric acid corner (avoidance behavior). This illustration clearly indicates that the amount of avoidance behavior induced by an odor is not connected to the amount of freezing induced by this odor.

DISCUSSION

The aim of the present study was to compare the aversion- and fear-inducing properties of TMT and butyric acid. First, we identified concentrations of TMT and butyric acid that induced an identical amount of avoidance behavior. These concentrations [5 µl 10^{-1} % vol. (3.87 µmol) TMT; 5 µl sheer butyric acid (54.7 µmol)] were then tested in exposure boxes for fear-inducing properties. Clearly, only TMT but not butyric acid or the solvent DEP was able to induce fear behavior as measured by freezing.

Avoidance behavior in rats and mice towards TMT (Blanchard et al., 2003; Hebb et al., 2004; McGregor et al., 2002; Wallace and Rosen, 2000), as well as avoidance behavior towards butyric acid (Hebb et al., 2004; Wallace and Rosen, 2000) has been reported several times. Compared with most of these studies, we observed avoidance behavior using lower concentrations of the odors, indicating that the setup and protocol we used was very sensitive to avoidance behavior. Most importantly, we could figure out concentrations of TMT and butyric acid that are similarly avoided. It should be noted that the used concentrations induced only moderate avoidance behavior whereas, by contrast, strong avoidance behavior was observed in the studies quoted above. By using odor

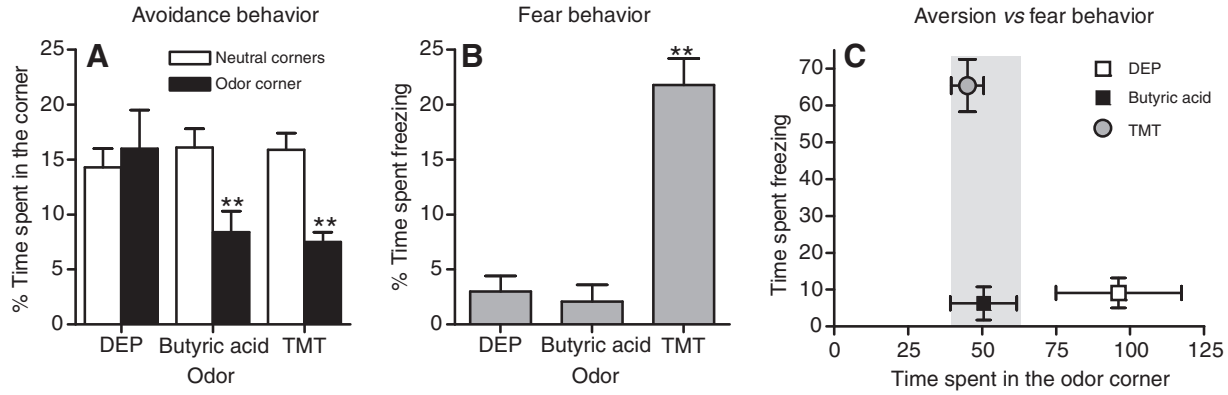

Fig. 1. (A) Means + s.e.m. (standard error of the mean) of the percentage of time the rats spent in the corners of the open field arena. (Open bars: mean time spent in the neutral corners; closed bars: time spent in the odor corner.) (B) Percentage time (means + s.e.m.) the rats spent freezing during exposure to TMT, butyric acid or the control (the solvent DEP). (C) Mean time (±s.e.m.) spent in the odor corner (avoidance experiment) and spent freezing (freezing experiment). The gray area shows the mean ± s.e.m. time spent in the butyric acid corner (avoidance behavior). **Indicates a *P*-value <0.01 (*post-hoc* pairwise comparison with control corner/odor).

concentrations inducing only moderate avoidance behavior, the risk of evoking unspecific responses, e.g. due to intense noxious properties of the odors, is reduced. This is additionally supported by the fact that locomotor behavior was not generally affected by TMT or butyric acid in our avoidance experiments. This stands in contrast to previous findings in another rodent species (Perrot-Sinal et al., 1996); however, we used very different odor concentrations.

The working hypothesis of the current study was that the fact that an odor induces avoidance behavior reveals no information about its fear-inducing potential. An odor can be avoided due to its aversive, noxious or repugnant properties or due to its fear-inducing properties. To determine whether an odor or the specific concentration that instigates its avoidance is also fear inducing, it must also induce other species-specific defensive reactions in the tested animal. One of these defensive responses of rats that is frequently used as an index of fear is freezing behavior (e.g. Blanchard and Blanchard, 1969; Blanchard et al., 2005; Bolles, 1970; Wallace and Rosen, 2000). Therefore, we tested the two similarly avoided amounts of TMT and butyric acid for their ability to induce freezing behavior. Very clearly only TMT but not butyric acid induced freezing behavior. It should be added that even higher amounts of butyric acid are unable to induce freezing behavior [(Wallace and Rosen, 2000) 1056 µl; (Morrow et al., 2000) 105 µl; (Hebb et al., 2004) 105 µl for mice] whereas freezing during exposure to TMT has been shown several times [summarized by Fendt et al. (Fendt et al., 2005)].

Here, in contrast to all previous studies, which tested the avoidance and fear-inducing properties of TMT and butyric acid, we directly compared the aversive properties of the odors with their fear-inducing properties. Most importantly, we identified weak concentrations of the odors (54.7 µmol butyric acid and 3.87 µmol TMT) inducing similar amounts of reliable avoidance behavior. This differs from the study of Wallace and Rosen (Wallace and Rosen, 2000) in which the avoidance- and fear-inducing properties of TMT and butyric acid was compared using very high doses (300 µmol). Here, a very strong avoidance response of the odor source was observed, and it is not impossible that such high concentrations lead to unspecific responses. However, the high butyric acid and caproic acid concentrations that were used by Wallace and Rosen (Wallace and Rosen, 2000) were also unable to induce freezing behavior. In conclusion, the results of the present study, together with the different data reported in the literature, clearly demonstrate that TMT as well as other odors have properties (aversive, repugnant or repellent) that induce avoidance responses. Most importantly, not all odors that induce avoidance behavior will also induce fear behavior (as demonstrated by the present study), clearly indicating that the avoidance-inducing properties of odors are not necessarily responsible for their fear-inducing properties.

In conclusion, we identified a low concentration of butyric acid and TMT to which the rats respond with a moderate but robust avoidance behavior. Despite these similar avoidance-inducing properties only TMT but not butyric acid was able to induce fear as measured by freezing. This clearly shows that the fear-inducing properties of TMT cannot simply be reduced to its avoidance-inducing properties and indicates that TMT, in fact, represents a predator odor with specific fear-inducing properties.

LIST OF ABBREVIATIONS

ANOVA	analysis of variances
DEP	diethylphthalate
TMT	2,4,5-trimethyl-3-thiazoline

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