Research report

Freezing to the predator odor 2,4,5 dihydro 2,5 trimethylthiazoline (TMT) is disrupted by olfactory bulb removal but not trigeminal deafferentation

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HIGHLIGHTS

• Rats were exposed to TMT following either olfactory bulb removal or trigeminal nerve transection.
• Subjects lacking facial trigeminal innervation show normal fear behavior to TMT.
• Rats without olfactory bulbs fail to respond to TMT, though they can show fear to other stimuli.
• We conclude that fear behavior to TMT is mediated by the olfactory systems.

ABSTRACT

2,4,5 dihydro 2,5 trimethylthiazoline (TMT) is a synthesized component of red fox anal secretions that reliably elicits defensive behaviors in rats and mice. TMT differs from other predator odors because it is a single molecule, it can be synthesized in large quantities, and the dose for exposure is highly controllable in an experimental setting. TMT has become a popular tool for studying the brain mechanisms that mediate innate fear behavior to olfactory stimuli. However, this view of TMT as a biologically relevant olfactory stimulus has been challenged by suggestions that the odor elicits fear behavior due to its irritating properties, presumably working through a nociceptive mechanism.

To address this criticism our lab measured freezing behavior in rats during exposures to 2 odors (TMT and butyric acid) and H2O (no odor control) following either surgical transection of the trigeminal nerves or ablation of the olfactory bulbs. Our findings (Experiment 1) indicate that freezing behavior to TMT requires an intact olfactory system, as indicated by the loss of freezing following olfactory bulb removal. Experiment 2 revealed that rats with trigeminal nerve transection freeze normally to TMT, suggesting the olfactory system mediates this behavior to TMT. A replication of Experiment 1 that included contextual fear conditioning revealed that the decreased freezing behavior was not due to an inability of olfactory bulb ablated rats to freeze (Experiment 3). Taken together, these findings support TMT’s role as an ecologically relevant predator odor useful in experiments of unconditioned fear that is mediated via olfaction and not nociception.

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1. Introduction

Predators and predator odors have become increasingly utilized to assess the neural mechanisms that mediate fear responses. Odors derived from cats, ferrets, weasels, and foxes have been shown to elicit a variety of innate defensive behaviors in rodents; including avoidance, reduction in locomotor activity, risk assessment, and freezing [2,8,15,21]. Until recently, most odors used in these experiments have been obtained from fur, hair, saliva, urine, and feces samples. However, synthetic predator odors originally isolated from bodily secretions can now be chemically synthesized for laboratory use. One odor in particular, 2,4,5 dihydro 2,5 trimethylthiazoline (TMT), a volatile compound originally isolated from the anal secretions of the red fox, induces robust freezing behavior in rats [9,17,26,31,33] and mice [7,13,14]. In an experimental setting TMT offers advantages over natural predator odors because it is a single molecule, its concentration can be precisely controlled, and it induces freezing in rats and mice in a dose-dependent manner [10,26,33].

Despite these advantages, TMT has received considerable criticism as to whether its behavioral effects are due to its olfactory...
properties. More specifically, it has been postulated that TMT induces fear behavior simply because it is a noxious, irritating substance [2,4,11,23]. It is also possible that it has both olfactory and noxious properties, since the olfactory system and the trigeminal nociceptive system are often both involved in the qualia of odor perception [4,10].

Predator odors are thought to induce behavioral responses through the activation of two olfactory pathways: one projecting from the nasal epithelium to the main olfactory bulb, and a second originating from the vomeronasal organ to the accessory olfactory bulb [19,20,22,28]. Alternatively, nociceptive information from the intranasal muscosa and the dorsal and tip of the nose is relayed via the frontal and ethmoidal branches of the ophthalmic division of the trigeminal nerve [32]. The ethmoidal branch provides central afferents for nociception from the nasal cavity, whereas the frontal branch provides afferents from the external surface of the snout. The infraorbital branch of the trigeminal nerve also is responsible for sensory information around the mouth region and whiskers [32]. These trigeminal pathways then project from the nasal cavity and frontal part of the face to the trigeminal sensory nucleus and subsequently innervate parts of the subnucleus caudalis and subnucleus interpolaris in the brainstem [1,32].

If TMT elicits fear responses through nociception it would therefore require a functional trigeminal pathway in order to produce freezing behavior. Conversely, if TMT is operating as an olfactory stimulus it would require intact olfactory bulbs to elicit freezing responses. The present study aimed to answer this question by selectively removing the main and accessory olfactory bulbs or portions of the facial trigeminal nerves and then testing subjects on their freezing behavior during exposure to H2O, TMT and butyric acid (BA). Butyric acid, a component of rancid butter with an unpleasant and acid smell, was chosen as a comparison stimulus since rodents avoid the compound, suggesting it stimulates the trigeminal nerve [9,13,33].

2. Methods

2.1. Subjects

A total of 60 male Sprague-Dawley rats obtained from Charles River breeders (Wilmington, MA) were used in the following studies. At the start of each experiment subjects were 90-120 days old and weighed between 300-350 g. All rats were pair-housed in opaque shoebox cages (20 cm × 46 cm × 23 cm), maintained on a 0700–1900 light/dark cycle, and given access to food and water ad libitum. Experimental sessions were conducted during the light phase, between 0800 and 1700 h. All procedures were in accordance with the US National Institutes of Health Guide for the Care and Use of Experimental Animals and were approved by the University of Delaware’s IACUC.

2.2. Apparatus

All experiments measuring freezing behavior during odorant presentation were conducted in four rectangular Plexiglas chambers (16.5 cm × 12.1 cm × 21.6 cm) with metal grid flooring (nine stainless steel bars 4 mm in diameter, spaced 1.0 cm apart). Each box was positioned on a Plexiglas frame inside a fume hood with illumination provided by an overhead light. A camera positioned approximately 2 ft from the chambers simultaneously recorded activity in each chamber and transmitted the signal to a nearby Dell Computer. Freezing behavior was recorded using FreezeFrame (Actimetrics Software) set to 4 chamber/1 mode and quantification of the behavior utilized the partner FreezeView (Actimetrics Software) software.

2.3. Surgical procedures

2.3.1. Olfactory bulb removal

The target of the ablation procedure was complete removal of both the main and accessory olfactory bulbs. Each rat was anesthetized via an intraperitoneal injection of a ketamine/xylazine cocktail (87 mg/13 mg/kg) and positioned in a stereotaxic surgical apparatus to ensure stability during the operation (Kopf Instruments, Tujunga, California). Incisions were made in the scalp, the skull surface was exposed, and a 5 mm2 window was drilled through the bone between 5 and 10 mm anterior to Bregma. After clearing of the meninges a bilateral aspiration of the olfactory bulbs was performed using a Pasteur pipette (Fisherbrand) attached to a trap and vacuum pump. Following this aspiration sterile Gelfoam was inserted into the cavity and the wound closed with VetBond. Subjects were allowed to heal for 7 days following surgery and their weights were consistently monitored. Sham surgeries consisted of making scalp incisions, exposing the skull surface, and closing the wound with VetBond. Buprenorphine (0.01 mg/kg, s.c.) was administered to manage pain and batriy (5.0 mg/kg, s.c) was given to inhibit infection.

2.3.2. Transection of the trigeminal nerve

One week following arrival in the animal colony each rat was anesthetized as above and positioned in a stereotaxic apparatus (Kopf Instruments, Tujunga, California). The surgical procedure for the transection of the infraorbital trigeminal nerve was adapted from Kinsant et al. [18]. Bilateral 7 mm anterior–posterior incisions were made 2 mm above each eye. Fascia and muscle were moved away from the bone using a periosteal elevator and the cuticle was retracted laterally. The frontal branch of the ophthalmic branch of the trigeminal nerve was transected during this procedure. Once retracted, a small surgical hook was used to reach and isolate the infraorbital and ethmoidal branches of the trigeminal nerve; these nerves were transected with a scissors. The wound was then sutured with Vicryl sutures (Ethicon, Inc.) and allowed to heal for 5 days. The procedure for the sham condition consisted of making the incisions, exposing the orbit, and suturing the skin in the same manner as was done for the transection of the nerve. Buprenorphine (0.01 mg/kg, s.c.) was administered to manage pain and butyryl (5.0 mg/kg, s.c.) was given to inhibit infection. To reduce post-surgery inflammation subjects were given ibuprofen (0.2 mg/mL) in their drinking water during the 5 recovery days.

2.4. Behavioral protocols

Odor exposure was conducted in an identical manner for all experiments. Prior to all subjects exposures were acclimated to the testing environment for 10 min per day for two days. Odor exposure was performed by adding liquid odorant to two pieces of filter paper (2 cm2) affixed to the left and right test chamber walls prior to placing the subject into the chamber. Recording of freezing behavior began immediately and continued for a total of 10 min per exposure session. The odorants and concentrations, and volume applied per exposure were as follows: TMT (300 µmole, 19.4 µL on each strip of paper), Butyric Acid (900 µmole, 39.6 µL on each strip of paper), and H2O (30 µL on each strip of paper). 300 µmole of TMT was chosen because this amount produces asymptotic levels of freezing behavior [33]. Since TMT is three times as volatile as butyric acid, three times as much butyric acid was used to produce equivalent levels of TMT and butyric acid exposure [17].

In Experiment 3 evaluation of shock-induced freezing was conducted through a 1-second central fear conditioning design. One day following TMT exposure subjects were allowed to acclimate in the testing chambers for 3 min before receiving a 1 s, 1.5 mA scrambled foot shock delivered through the metal grid floor. Recording of freezing behavior continued for 4 min following shock delivery before subjects were returned to their home cages. Subjects in Experiment 3 were not exposed to butyric acid or water.

Following each test, subjects were returned to their home cage and the test chambers were cleaned with a 5% ammonium hydroxide solution. Due to the volatile nature of the odorants a minimum interval of 15 min occurred between each session to ensure ventilation of the chambers and testing room. For all experiments, freezing behavior was defined as the cessation of all bodily movement except for breathing [3]. In all analyses freezing bout length was set to a minimum of 0.75 s and freezing threshold was set based upon the instructions of the FreezeFrame software. Behavior was analyzed with mix-model analysis of variance and Bonferroni corrected t-tests using SPSS software (version 16).

2.5. Histology

2.5.1. Olfactory bulb removal

Following all behavioral tests subjects were euthanized via rapid decapitation and the brain was harvested, flash frozen in dry ice chilled 2-methylbutane, and stored in a –800 C freezer. Evaluation of the olfactory bulb lesion was performed visually by photographing each subject’s brain and inspecting them for completeness of the removal. Subjects with incomplete olfactory bulb removal or whose ablation included frontal cortical tissue were excluded for the analyses.

2.5.2. Trigeminal transection

Following all testing procedures all subjects were overdosed with a ketamine/xylazine cocktail (87 mg/13 mg/kg, i.p.) and perfused intracardially with 0.9% saline. Verification of transected ethmoidal and infraorbital branches of the trigeminal nerve was performed visually by removing the eye, skin and tissue around the orbit and visually inspecting whether the nerve was cut or remained intact. Any subject with an intact or partial transection of infraorbital and ethmoidal nerve branches was eliminated from the data pool.
3. Results

3.1. Experiment 1: Ablation of the olfactory bulbs reduces freezing to TMT

Post-mortem examination of the olfactory bulb lesions revealed successful bilateral aspiration of the olfactory bulbs in 6 of 9 subjects. Subjects with incomplete aspiration of the olfactory bulbs were removed from the analyses so that a total of 15 subjects remained, 6 OB-lesioned and 9 shams. See Fig. 1 for representative depictions of Sham and OB-lesioned brains.

Subjects were tested on freezing to H2O, TMT and Butyric Acid (BA) in independent 10 min exposure sessions (Fig. 2). A mixed model Analysis of Variance comparing odor (H2O, TMT, BA) and surgery conditions (Olfactory bulb lesion and Sham) reveal a significant main effect of odor ($F_{1,13} = 28.01; p < 0.001$), no main effect of condition ($F_{1,13} = 2.55; \text{n.s.}$), yet a significant odor by surgery condition interaction effect ($F_{1,13} = 21.81; p < 0.001$).

Bonferroni corrected t-tests were then used to examine each level of odor both within and between the surgery conditions. These analyses revealed that Sham rats froze more to TMT than to H2O ($p < 0.001$) and BA ($p < 0.001$), but freezing to BA and H2O did not differ from each other. Freezing to TMT in olfactory bulb lesioned rats (OB-lesion) was significantly reduced compared to Sham rats ($p < 0.001$), but freezing to H2O and BA did not differ between OB-lesion and Sham rats.

3.2. Experiment 2: Trigeminal nerve transection does not affect freezing to TMT

Post-mortem verification of the trigeminal nerve lesion revealed successful bilateral transection of the infraorbital and ethmoidal branches in 14 of 20 subjects. Subjects without complete transection of the trigeminal nerve were removed from all analyses. In total, 28 subjects were used in the analyses, 14 trigeminal transection and 14 shams. Fig. 3 shows examples of the intact and transected nerves observed during the visual inspection process.

Subjects were tested on freezing to H2O, TMT, and BA in independent 10 min exposure sessions (Fig. 4). A mixed model
analysis of variance comparing odor (H₂O, TMT, BA) and surgery conditions (Trigeminal transection and Sham) revealed a significant main effect of odor (F₁,26 = 170.11; p < 0.0001), a significant main effect of surgery condition (F₁,26 = 5.17; p < 0.05) and a significant interaction between odor and surgery (F₁, 26 = 11.85; p < 0.05).

Bonferroni corrected t-tests revealed that both sham and trigeminal transection subjects froze to TMT more than to H₂O (both p < 0.001) and BA (both p < 0.001). However, sham and trigeminal transected rats did not differ in their amount of freezing during TMT or H₂O presentation. Interestingly, sham subjects froze significantly more to BA than trigeminal transection subjects (p < 0.001) and also significantly more to BA than to H₂O (p < 0.001). These analyses reveal that the trigeminal nerve transections had no effect on freezing during H₂O or TMT, but the transections reduced freezing behavior to BA exposure.

3.3. Experiment 3: Reduced freezing to TMT following olfactory bulb removal is not due to a performance decrement

The reductions in freezing behavior seen in Experiment 1 led to questions regarding whether the ablation of the olfactory bulbs altered a subjects’ ability to freeze. Rats lacking olfactory bulbs display increased locomotor activity (For a review, see Song and Leonard [29]) which could contribute to the decreased freezing behavior observed in Experiment 1. In order to test this hypothesis a small replication was performed to evaluate whether subjects lacking olfactory bulbs display normal freezing behavior following a footshock, as observed during standard contextual fear-conditioning.

Olfactory bulb aspiration surgery was performed in 4 rats, with 2 successful aspirations being used in the final analyses. A total of 6 subjects (2 olfactory bulb lesioned, 4 shams) were tested in independent sessions for freezing to H₂O, TMT, and following a footshock (Fig. 5). A mixed model Analysis of Variance comparing trial (H₂O, TMT, Post-shock) and surgery condition (olfactory bulb removal and sham) revealed a significant main effect of trial (F₁,4 = 15.006; p < 0.05), no effect of condition (F₁,4 = 1.122; n.s.), and a significant trial by condition interaction (F₁,4 = 31.913; p < 0.005). Bonferroni corrected t-tests revealed that olfactory bulb removal subjects froze significantly less than shams to TMT (p < 0.05), but do not differ from shams in their post-shock freezing.

4. Discussion

A number of studies have challenged the notion that fear behavior evoked by TMT is due to it being a biologically relevant olfactory stimulus. Rather, it has been suggested that the fear and anxious behaviors seen during presentation of TMT are due to the compound’s irritant properties, presumably mediated through the trigeminal sensory system [2,4,11,23]. We approached this question by removing the entire olfactory bulbs, which would eliminate odor sensation, or transection of the ethmoidal, frontal and infraorbital branches of the trigeminal nerve, which would reduce noxious and nociceptive sensations from the nasal cavity, snout and whiskers. The primary findings reveal that rats with ablated olfactory bulbs failed to display freezing behavior to TMT (Fig. 2), but this deficit was not due to an inability to perform freezing behavior as OB lesioned rats displayed normal levels of shock induced freezing (Fig. 5). In contrast, rats lacking substantial facial somatosensory afferents due to trigeminal nerve transection exhibited intact freezing behavior in response to TMT (Fig. 4).

Furthermore, we compared freezing to TMT with freezing to BA exposure, an odor which rats and mice avoid but does not evoke significant levels of freezing behavior [9,16,27,33]. However, in Experiment 2 sham subjects displayed significantly high levels of freezing to BA (a mean of 58% time spent freezing), and at the same high levels that TMT exposure produced. It is possible that the sham surgery could have sensitized the rats to the irritating properties of BA, resulting in the significantly higher levels of freezing (Fig. 4). Interestingly, the high levels of freezing to BA were attenuated in rats with trigeminal transections suggesting that the irritating noxious properties of BA drove the increases in freezing in the sham rats. Importantly, the same trigeminal nerve transected rats did not display deficits in freezing to TMT. There is little known about which sensory system butyric acid engages; however this finding indicates significant involvement of the trigeminal system in detecting the compound’s potential irritant properties.

Hacquemard et al. [14] demonstrated that transitory destruction of mouse olfactory epithelium and neurons via ZnSO₄ nasal perfusion resulted in diminished avoidance and freezing behavior during both TMT and natural fox feces exposure. However, the ZnSO₄ treatment produced no behavioral deficits during exposure to the noxious yet presumably an ecologically irrelevant odor, toluene. A similar study by Galliot et al. [13] found an interesting contrast in which the ZnSO₄ treatment reduced freezing behavior but did not affect avoidance. Collectively the authors conclude that freezing to TMT is mediated primarily by the olfactory system.
though the trigeminal system may provide a small contribution. Taken together, the findings of both these studies and ours suggest that TMT acts as a fear inducing stimulus through its effects on the olfactory systems. Any irritant properties of the compound mediated by the trigeminal system do not appear to be sufficient in evoking freezing behavior.

This interpretation, that TMT evoked freezing is mediated by the olfactory systems, does not answer the question of which olfactory system is important for this response. The main olfactory system mediates perception of volatile, airborne chemicals [6], while the accessory olfactory system mediates innate responses to non-volatile, fluid-phase chemicals, typically pheromonal cues [5,24]. While natural predator odors induce Fos protein expression in both the main [8,12,30] and accessory [8,22,25] olfactory bulbs of rodents, relatively few experiments have successfully delineated unique functional roles for each system during predator odor exposure.

Recent work by Masini and colleagues [22] attempted to reveal such a functional dissociation between the main and accessory olfactory systems by testing rats’ responses to ferret odor following selective destruction of the olfactory epithelium or vomeronasal organ. Their primary findings, that disruption of the endocrine response to ferret odors required the combined destruction of both olfactory organs. However, Masini et al. [22] also found that the combined destruction of the olfactory organs resulted in a non-specific disruption of defensive behavior to the odors tested, whereas our behavioral data indicated a decrement specific to TMT exposure after both main and accessory olfactory bulb removal, but not to BA. It is possible that this contrast in behavioral results is related to the difference between natural ferret odor, which may have volatile and non-volatile components, and TMT, which is highly volatile, and only the main olfactory system might be necessary for its fear-inducing properties.

In an important study, Kobayakawa and colleagues [20] developed knockout mice with dorsal epithelial zone deletion of olfactory neurons to determine whether the dorsal or ventral subdivisions of the main olfactory bulb serve different functional roles in mediating responses to TMT. The authors found that mice lacking functional glomerular structures within the dorsal domain failed to show avoidance behavior to TMT even though they could still detect and use TMT as a conditioned stimulus during a discrimination task. These same subjects showed avoidance responses to other aversive odors, indicating that the change in behavior was specific to TMT. They concluded that the main olfactory bulb has distinct dorsal and ventral territories responsible for mediating innate responses to odors (i.e., TMT) and for associative learning of odor information, respectively [20].

These findings are in agreement with TMT’s volatile properties and its lack of solubility in aqueous solutions. Fos expression in rats also suggests that the main olfactory bulb is preferentially involved in transduction of odorant properties of TMT, as TMT exposure has been shown to induce Fos expression in the main olfactory bulb, but not the accessory olfactory bulb [30]. These collective findings suggest that TMT’s fear inducing properties are mediated by the main olfactory system.

In a similar vein to the gross destruction of olfactory systems, we transected the frontal, ethmoidal, and infraorbital branches of the trigeminal nerve, obscuring the specific branches responsible for the decrease in freezing to BA. The ethmoidal branch provides central afferents for nociception from the nasal cavity, whereas the frontal branch provides afferents from the external surface of the snout. The infraorbital branch transduces touch and position information from the whiskers [32]. Based on the known functions of the branches, we surmise that the ethmoidal carries most of the nociceptor stimulation of BA. Extensive studies with selective removal of various combinations of these different branches would be required to determine which branch or branches is most involved in the response to BA.

In summary, we found that complete removal of the olfactory systems led to loss of freezing behavior to TMT. This ablation did not affect responding to the pungent odorant butyric acid and was not due to an inability to perform freezing behavior. In sharp contrast, however, is the finding that trigeminal denervation did not affect freezing to TMT but instead selectively attenuated freezing to BA. This dissociation provides strong evidence that TMT evokes freezing behavior through an innate defensive response initiated in the olfactory systems, rather than through pain mechanisms.

Acknowledgements

Research for this work was supported by University of Delaware. We would like to thank our undergraduate research assistants Andrew Agostini, Wojtek Domoycz, Kathryn O’Connell, Blen Weldekidan, and Kristin Gagliardi, for help in running and analyzing some of the behavioral studies.

References


