Trimethylthiazoline supports conditioned flavor avoidance and activates visceral sensory, hypothalamic, and limbic circuits in rats

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Myers, Elizabeth A., and Linda Rinaman. Trimethylthiazoline supports conditioned flavor avoidance and activates visceral sensory, hypothalamic, and limbic circuits in rats. Am J Physiol Regul Integr Comp Physiol 288: R1716–R1726, 2005. First published January 20, 2005; doi:10.1152/ajpregu.00479.2004.—Interoceptive stimuli modulate stress responses and emotional state, in part, via ascending visceral sensory inputs to the hypothalamus and limbic forebrain. It is unclear whether similar visceral sensory pathways are recruited by emotionally salient exteroceptive stimuli, such as odors. To address this question, we investigated conditioned avoidance and central c-Fos activation patterns in rats exposed to synthetic trimethylthiazoline (TMT), an odoriferous natural component of fox feces. Experiment 1 demonstrated that rats avoid consuming novel flavors that previously were paired with TMT exposure, evidence that TMT supports conditioned flavor avoidance. Experiment 2 examined central neural systems activated by TMT. Odor-naive rats were acutely exposed to low or high levels of TMT or a novel nonaversive control odor and were perfused with fixative 60–90 min later. A subset of rats received retrograde neural tracer injections into the central nucleus of the amygdala (CeA) 7–10 days before odor exposure and perfusion. Brain sections were processed for dual-immunocytochemical detection of c-Fos and other markers to identify noradrenergic (NA) neurons, corticotropin-releasing hormone (CRH) neurons, and retrogradely labeled neurons projecting to the CeA. Significantly greater proportions of mediadullary and pontine NA neurons, hypothalamic CRH neurons, and CeA-projecting neurons were activated in rats exposed to TMT compared with activation in rats exposed to the nonaversive control odor. Thus the ability of TMT to support conditioned avoidance behavior is correlated with significant odor-induced recruitment of hypothalamic CRH neurons and brain stem visceral sensory inputs to the CeA.

c-Fos; noradrenergic; corticotropin-releasing hormone; paraventricular nucleus of the hypothalamus; central nucleus of the amygdala; nucleus of the solitary tract; parabrachial nucleus; ventrolateral medulla

STRESS AND ANXIETY RESPONSES in humans and animals include somatic, autonomic, and neuroendocrine components and can be induced by a wide variety of interoceptive and exteroceptive stimuli in natural and experimental conditions. Many researchers have sought to use ethologically relevant stress and anxiety paradigms in experimental animals to probe the central neural mediation of species-specific behavioral and physiological responses (2, 6, 9, 10, 23). For example, olfactory cues exert a powerful influence on rodent behavior; thus odors such as trimethylthiazoline (TMT) may provide a useful experimental model with which to examine stimulus-evoked behavioral and physiological responses and their central neural mediators.

TMT is a volatile sulfur-containing compound isolated from fox feces. Results from behavioral studies support the view that TMT is repellent, noxious, and/or aversive to rats and may serve as a partial predator cue. Acute or repeated exposure of laboratory rats to TMT reduces food intake (3), elevates plasma levels of ACTH and corticosterone (6, 27, 32), and can elicit unconditioned freezing behavior (11, 45). TMT also promotes conditioned avoidance responses in wild rats that are naive to foxes (43). Although acute or repeated exposure of rats to TMT consistently evokes unconditioned avoidance responses, three studies have reported that TMT does not promote contextual fear conditioning (2, 23, 45). It is unclear, however, whether TMT can serve as the unconditioned stimulus for other types of emotional learning, such as conditioned flavor avoidance (CFA) (29). Rats learn to avoid contact with tastants, odors, and flavors (i.e., combined taste and odor stimuli) when such stimuli are temporally paired with nausea and malaise. Rats also learn to avoid tastants, odors, and flavors that have been paired with nonnauseogenic treatments such as foot shock (31) or colonic distension (30), presumably because of the anxiogenic, fear-inducing, or otherwise aversive nature of such treatments.

The present study confirms the aversive nature of TMT by demonstrating that it can serve as an unconditioned stimulus that supports CFA behavior in rats. We also have examined potential neural correlates of TMT-induced aversion by characterizing and comparing central patterns of neural c-Fos protein expression in rats after acute exposure to TMT or a demonstrably nonaversive control odor, with a specific focus on odor-induced recruitment of brain stem noradrenergic (NA) neurons, hypothalamic corticotropin-releasing hormone (CRH)-positive neurons, and medullary and pontine neurons with axonal inputs to the central nucleus of the amygdala (CeA).

MATERIAL AND METHODS

Animals

Adult male Sprague-Dawley rats (250–320 g body wt; Harlan Laboratories) were housed singly in stainless steel cages in a controlled environment (20–22°C, 12:12-h light-dark cycle, lights on at 0700) with ad libitum access to water and pelleted chow (Purina 5001), except as noted. All experimental protocols were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Study 1: Conditioned Flavor Avoidance

Twelve rats (~300 g body wt) in two experimental cohorts (n = 6 rats/cohort) were used to determine whether acute exposure to TMT...
or a novel control odor (banana extract, BE) supports the formation of CFA. For this purpose, a sensitive two-bottle choice procedure (8) was used to generate preference ratios for odor-paired vs. non-odor-paired flavors. The aversive potentials of TMT and BE were examined in separate consecutive CFA experiments spaced 4 days apart within each experimental cohort. One cohort of rats was first trained and tested for conditioned avoidance of TMT-paired flavors. Training and testing for conditioned avoidance of BE-paired flavors was initiated 4 days later. The experimental order was reversed in the second cohort of rats such that training and testing for conditioned avoidance of BE-paired flavors was examined first, followed by training and testing for conditioned avoidance of TMT-paired flavors. All CFA training and testing procedures were conducted during the light cycle of the photoperiod, between 1500 and 1700.

Training and testing for conditioned avoidance of TMT-paired flavors. Rats underwent 22 h of water deprivation and were then transported to a quiet, ventilated training/testing room and acclimated to novel plastic shoebox cages (19 × 29 × 13 cm) with a wire top and Sani-Chip bedding. After 30 min, rats were presented with a single simulated cylinder (tap water containing 0.5% almond, vanilla, coconut, or brandy flavor extract (McCormick extract). The first experimental cohort received either almond (n = 3)- or vanilla-flavored tap water (n = 3). The second cohort received either brandy (n = 3)- or coconut-flavored water (n = 3). The left-right position of the single bottle on each cage was switched after 15 min, with cumulative fluid intake recorded at 15 and 30 min. Rats remained in the training/testing room in the same cages for 45 min after the end of fluid access; this training day provided the “non-odor-paired flavor” condition. Rats then were returned to their home cages and given 24 h of free access to water. Rats were then water deprived again for 22 h, transported to the training/testing room, and placed into shoebox cages containing milled corn cob bedding. After 30 min, they were presented with water containing the alternate flavor received by rats within their experimental cohort (i.e., vanilla if they received almond in the non-odor-paired condition, and vice versa; coconut if they received brandy in the non-odor-paired condition, and vice versa). Left-right bottle position was switched after 15 min, and cumulative intake was recorded at 15 and 30 min. Thirty minutes after the end of fluid access (CS-US interval based on Ref. 41), rats were exposed to TMT (PhenoTech; 40 μl neat-pipetted onto filter paper on top of the wire cage top) for 15 min. This provided the “TMT-paired flavor” condition. Rats were then returned to their home cages with water available ad libitum. After each odor exposure session, cages and wire tops were cleaned thoroughly with a germicidal detergent and deodorant (Quatricide PV; Pharmacal Research Laboratories) and allowed to air dry for at least 24 h before reuse to reduce residual odor traces.

Before the two-bottle choice testing day, rats were water deprived for 22 h and then transported to the training/testing room and given 30-min simultaneous access to two bottles containing the novel flavors encountered during the non-odor-paired and TMT-paired training conditions (i.e., vanilla and almond or brandy and coconut, depending on experimental cohort). A mixture of Sani-Chips and corn cob bedding was used in the training/testing cages to limit the possible influence of contextual conditioning effects on fluid intake during the two-bottle choice test. Bottle positions on each cage were switched after 15 min, with cumulative intakes of each flavor recorded at 15 and 30 min. Rats were then returned to their home cages with ad libitum water access.

Training and testing for conditioned avoidance of BE-paired flavors. A separate CFA experiment was conducted in both cohorts of rats by using BE as the unconditioned odor stimulus. In one experimental cohort, the BE experiment was initiated 4 days after the TMT experiment was completed. In the second cohort, the BE experiment was completed 4 days before the TMT experiment was initiated. The cohort that was trained and tested with 0.5% vanilla- and almond-flavored water in the TMT experiment was trained and tested with 0.5% brandy- and coconut-flavored water in the BE experiment, and vice versa. Training and testing procedures for conditioned avoidance of BE-paired flavors were similar to procedures described for the TMT experiment except that rats were exposed to BE (40 μl neat on filter paper; McCormick extract) instead of TMT in the “odor-paired flavor” condition.

CFA data analysis. Flavor preference ratios within each CFA experiment (i.e., TMT or BE) were determined by dividing the volume of odor-paired vs. non-odor-paired flavors consumed by the total volume consumed from both bottles during the 30-min choice test. Outcomes indicating flavor preference ratios of close to 50:50 were interpreted as an absence of CFA, whereas outcomes indicating significantly shifted preference ratios (e.g., 30:70) were interpreted as evidence for conditioned avoidance of the flavor represented by the lower value in the ratio. Preference ratio data from individual rats were averaged to obtain group preference ratios (means ± SE) within each experiment. Student’s t-test was used to determine whether group preference ratios for odor-paired and non-odor-paired flavors were significantly different, with significance set at P < 0.05.

Study 2: Odor-Induced c-Fos Expression

New groups of rats naive to TMT and BE were acclimated to daily handling for approximately 1 wk. On the day of acute odor exposure and death, rats was transported to a new quiet room and placed individually into 19 × 29 × 13-cm wire-topped shoebox plastic cages with Sani-Chip bedding. After a 30-min acclimation period, rats were exposed for 15 min to either TMT or BE. In each case, 40 μl of TMT or BE was pipetted onto a small piece of filter paper placed onto the wire top of each cage. Exposure of only one treatment group to one odorant was carried out in a single day to avoid odor contamination across groups. The odor exposure room was ventilated and under negative air pressure relative to the hallway to avoid odor dissemination to other areas. To determine whether odorant concentration (i.e., exposure level) affected neural activation patterns, we carried out odor exposure out under two conditions: 1) cages were placed in an area adjacent to a quiet, operating fume hood (low odor exposure), or 2) cages were placed in an area without a fume hood (high odor exposure). After 15 min of odor exposure, filter papers were removed from the cage tops, Rats were returned to their home cages for 90 min and then anesthetized and perfused transcardially with fixative (described in Perfusion and tissue preparation). Experimental outcomes revealed that the BE “low odor exposure” condition was associated with very little or no c-Fos expression in the brain regions of interest in this study, including retrogradely labeled neurons projecting to the CeA, similar to the low levels of c-Fos immunolabeling observed in control rats in our earlier studies (for example, see Refs. 28, 36, and 38). Thus, given the exceedingly low to absent c-Fos expression in this group of rats, we considered it unnecessary to include an additional group of control rats that were handled similarly but not exposed to BE or TMT.

Neural tracer injections. A subset of rats received bilateral, stereotaxically guided microinjections of retrograde neural tracer into the CeA 7–10 days before acute odor exposure and perfusion. For tracer injections, rats were anesthetized with halothane (1–3% in oxygen; Halocarbon Laboratories) and mounted into a stereotaxic frame. Two retrograde tracers were used: FluoroGold (FG; 2% in 150 mM NaCl; Fluorochrome) and cholera toxin β-subunit (CTb; 0.25% in 150 mM NaCl; List Biological Laboratories). Rats received FG injections targeted to the left CeA and CTb injections targeted to the right CeA. The dual tracer injection procedure increased the chance of obtaining at least one accurate tracer injection site in each rat. A 1.0-μl Hamilton syringe filled with either FG or CTb was attached to the stereotaxic arm. CeA coordinates (2.2 mm posterior, 4.0 mm lateral, and 8.2 mm ventral to bregma) were selected on the basis of a standard rat brain atlas (3). The syringe was lowered into the brain and left in place for 5 min before injection. FG (50 nl) or CTb (100–150 nl) was delivered by pressure over a 1- to 2-min period. The syringe
was left in place for an additional 7 min after each injection to reduce tracer diffusion up the needle tract. The skin was closed with stainless steel clips, a topical anesthetic (2% lidocaine) was applied to the incision site, and rats were returned to their home cages after recovery from anesthesia. Rats were given 7–10 days to recover from surgery and to allow sufficient time for retrograde tracer transport before acute exposure to either TMT or BE.

**Perfusion and tissue preparation.** Rats were deeply anesthetized with sodium pentobarbital (Nembutal; 50 mg/ip) and perfused transcardially with 50–100 ml of 150 mM NaCl followed by 500 ml of fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer with lysine and sodium metaperiodate) (24). Brains were post-fixed overnight at 4°C and then blocked and cryoprotected in aqueous 20% sucrose before sectioning.

**Histology and immunocytochemistry.** Coronal 35-μm tissue sections were cut from the caudal extent of the nucleus of the solitary tract (NST) through the rostral extent of the corpus callosum by using a freezing microtome. Sections were collected serially in six adjacent sets and stored at −20°C in cryopreservant (46). Sections were removed from storage and rinsed for 1 h in buffer (0.1 M sodium phosphate, pH 7.4) before immunocytochemical procedures were carried out. Antisera were diluted in buffer containing 0.3% Triton-X and 1% normal donkey serum. Biotinylated secondary antisera (Jackson Immunochemicals) were used at a dilution of 1:500.

Tissue sections were processed for immunocytochemical localization of c-Fos protein by using a rabbit polyclonal antiserum (1:50,000; provided by Dr. Philip Larsen, Rheoscience, Denmark) and Vectastain Elite ABC immunoperoxidase reagents (Vector Laboratories). The specificity of this antibody for c-Fos protein has been reported (37). Sections were processed using a nickel sulfate-intensified diaminobenzidine (DAB) reaction to generate a blue-black c-Fos reaction product in the nuclei of activated cells. In tracer-injected rats, two sets of c-Fos-labeled tissue sections were subsequently processed for immunoperoxidase localization of FG (rabbit anti-FG; 1:30,000 dilution; Chemicon) or CTb (goat anti-CTb; 1:50,000 dilution; List Biological Laboratories). DAB was used to generate a brown reaction product at the amygdala tracer injection sites and in the cytoplasm of retrogradely labeled neurons. In all rats, other sets of c-Fos-labeled sections were processed for dual-immunoperoxidase localization of cytoplasmic CRH (rabbit anti-CRH; 1:15,000 dilution; Penninsula) in the forebrain and of the NA synthetic enzyme dopamine β-hydroxylase (mouse anti-DH; 1:30,000 dilution; Chemicon) in the brain stem. After immunocytochemical processing, tissue sections were mounted onto Superfrost Plus microscope slides (Fisher Scientific), allowed to air dry overnight, dehydrated and defatted in graded ethanols and xylene, and coverslipped using Cytoseal 60 (VWR).

**Quantitative analysis.** Dual-immunoperoxidase-labeled tissue sections were analyzed with a light microscope to determine the proportion of phenotypically identified neurons activated to express c-Fos. Criteria for counting a neuron as retrogradely labeled (i.e., CTb or FG positive), DbH positive, or CRH positive included the presence of brown cytoplasmic immunoreactivity and a visible nucleus. Neurons were considered c-Fos positive if their nucleus contained blue-black immunoreactivity, regardless of intensity, and c-Fos negative if their brown cytoplasmic immunoreactivity and a visible nucleus. Neurons positive), DbH positive, or CRH positive included the presence of immunolabel, regardless of intensity, and c-Fos negative if their
development of CRH-positive paraventricular nucleus (PVN) neurons and the proportion activated to express c-Fos were documented at ×100 magnification by using an oil-immersion objective with the assistance of Stereo Investigator X-Y plotting software (MicroBrightField). CRH-positive neurons in the PVN were summed bilaterally and averaged in each rat across two sections spaced 210 μm apart that contained the highest density of CRH neural labeling. Activated CRH neurons (i.e., those with visible nuclear c-Fos labeling) were expressed as a percentage of the total number of CRH-positive neurons counted within the PVN.

Quantitative analysis of c-Fos expression by retrogradely labeled neurons and DbH-positive NA neurons was performed in brain stem regions that provide direct input to the CeA, including the pontine locus coeruleus (LC; location of the A6 NA cell group), the lateral parabrachial nucleus (PNB), the NST (location of the A2/C2 cell groups), and the caudal ventrolateral medulla (VLM; location of the A1/C1 cell groups). Retrogradely labeled or DbH-positive neurons were counted bilaterally in each region at ×40 magnification. Counts of retrogradely labeled DbH-positive neurons in the LC were summed bilaterally across 6–11 tissue sections spaced 210 μm apart in each rat. Retrogradely labeled neurons within the external portion of the lateral PBN were summed unilaterally and averaged over two sections spaced 210 μm apart that contained the highest density of retrograde labeling ipsilateral to the CeA tracer injection site in each rat. Counts of retrogradely labeled and DbH-positive NST and VLM neurons were grouped according to three rostrocaudal levels of the visceral NST defined with respect to the area postrema (AP); 1) sections caudal to the AP (caudal NST (cNST) and VLM (cVLM)); 2) sections at the level of the AP (mid NST (mNST) and VLM (mVLM)); and 3) sections rostral to the AP (rostal NST (rNST) and VLM (rVLM)). Counts in rostral sections were discontinued at the level at which the NST moves laterally away from the floor of the fourth ventricle. Retrogradely labeled NST and VLM neurons were counted at the same rostrocaudal levels at which activation of NA neurons was assessed.

Odor-induced activation of phenotypically defined neurons was examined using two- or three-way repeated-measures ANOVA to reveal significant overall main effects of odor, intensity, and/or brain subregion, with the use of Fisher’s least significant difference protected t-tests for multiple post hoc comparisons. Differences were considered statistically significant when \( P < 0.05 \).

**RESULTS**

**Study 1: CFA**

All rats consumed at least 12 ml of each novel flavor during initial 30-min, one-bottle training sessions (average: 18.3 ± 1.5 ml; range: 12.5–21.0 ml). Cumulative fluid intakes during one-bottle training sessions did not differ significantly as a function of flavor (i.e., vanilla vs. almond vs. coconut vs. brandy), flavor presentation order (i.e., vanilla and almond in the first CFA experiment followed by coconut and brandy in the second CFA experiment, or vice versa), or order of experimental testing (i.e., TMT experiment first followed by BE experiment, or vice versa).

Cumulative 30-min intake volumes during two-bottle choice tests were analyzed using Student’s t-test to determine whether group mean preference ratios for non-odor-paired flavors vs. odor-paired flavors were shifted significantly from 50:50, which is the expected ratio in the absence of a conditioning effect. Results from TMT and BE experiments were analyzed separately.

Both experimental cohorts demonstrated significant avoidance of flavors previously paired with TMT exposure (Fig. 1A). Preference ratios were not significantly different between co-
horts, providing evidence that experimental outcome was not affected by training and testing order (i.e., TMT experiment first followed by BE experiment, or vice versa) and also was not affected by the flavor used for TMT conditioning (i.e., vanilla or almond vs. brandy or coconut). After data from both experimental cohorts of rats were combined ($n = 12$), fluid intake in the 30-min, two-bottle choice test consisted of 14.1 ± 1.4 ml of flavors paired with no odor and 5.9 ± 1.6 ml of flavors paired with TMT ($P < 0.05$). These results indicate a conditioned avoidance of TMT-paired flavors (which comprised only 29.6 ± 7.9% of total fluid intake; $n = 12$) and a shifted preference toward non-odor-paired flavors (which comprised 69.4 ± 7.1% of total fluid intake; $n = 12$).

In contrast to the relatively strong conditioned avoidance of TMT-paired flavors, neither cohort of rats avoided consuming flavors previously paired with BE exposure ($B$).

Across experiments, the average intake of BE-paired flavors (10.3 ± 1.5 ml; $n = 12$) was significantly greater ($P < 0.05$) than the average intake of TMT-paired flavors (5.9 ± 1.6 ml; $n = 12$) in separate two-bottle choice tests. These results support the prediction that a preference for BE-paired flavors over TMT-paired flavors would have been revealed if rats had been given simultaneous access to flavors previously paired with each odorant.

**Study 2: Odor-Induced c-Fos Expression**

Activation of DbH-positive NA neurons in NST (A2/C2 cell groups). Three-way repeated-measures ANOVA revealed significant main effects between subjects of odor intensity (low vs. high) [$F(1,37) = 172.3, P < 0.0001$] and odor type (BE vs. TMT) [$F(1,37) = 5.7, P = 0.02$] on activation of NA neurons in the NST, with high exposure levels of TMT and BE activating significantly more NA neurons per tissue section than low exposure levels of the corresponding odorants ($P < 0.05$ for each within-odor comparison; Fig. 2A). There was no

cohorts indicates that the outcome was not affected by either the training and testing order for the TMT and BE experiments or the flavors used for conditioning.

**Fig. 1.** Average group preference ratios for novel flavors in 2-bottle choice tests after flavors were previously paired with no odor vs. trimethylthiazoline (TMT) ($A$) or with no odor vs. banana extract (BE) ($B$). Results from 2 experimental cohorts of rats are shown. Dashed lines indicate expected preference ratios (50%) with no effect of flavor pairing condition. In $A$, intake of TMT-paired flavors is avoided relative to intake of no odor-paired flavors in both cohorts ($P < 0.05$ for TMT-paired flavor vs. no odor-paired flavor). Conversely, rats neither avoided nor preferred flavors previously paired with BE exposure ($B$).

**Fig. 2.** Odor-induced activation of noradrenergic (NA) neurons within the nucleus of the solitary tract (NST) (A2/C2 cell groups; $A$) and ventrolateral medulla (VLM) (A1/C1 cell groups; $B$) at levels caudal to the area postrema (AP) ($cNST$ and $cVLM$), levels containing the AP (mNST and mVLM), and levels rostral to the AP ($rNST$ and $rVLM$). TMT activated significantly more NA neurons per tissue section than BE ($P < 0.05$ at the indicated odor intensity levels. Group sizes ($n$) are indicated in legend.
significant interaction between odor intensity and odor type. Within subjects, there was a significant effect of rostrocaudal level (cNST, mNST, rNST) \(F(2,76) = 48.1, P < 0.0001\) and a significant interaction between odor intensity and rostrocaudal level on NA neuron activation \(F(2,76) = 35.5, P < 0.0001\). There was no significant interaction between odor type and rostrocaudal level. At all 3 rostrocaudal levels of the NST, activation of NA cells was significantly greater in rats after low TMT exposure compared with activation after low BE exposure (Fig. 2A). High TMT exposure activated significantly more NA neurons in the rNST compared with high BE exposure (Fig. 2A). Interestingly, however, activation of NA cells at cNST and mNST levels was similar in rats after high BE and high TMT exposure. Figure 3 shows representative photomicrographs of c-Fos activation at mNST levels in rats after exposure to low (Fig. 3A) or high levels of TMT (Fig. 3D).

**Activation of DbH-positive NA neurons in VLM (A1/C1 cell groups).** Similar to the NST results described, three-way repeated-measures ANOVA revealed significant main effects between subjects of odor intensity (low vs. high) \(F(1,37) = 418.5, P < 0.0001\) and odor type (BE vs. TMT) \(F(1,37) = 22.9, P < 0.0001\) on NA neuron activation in the VLM, with high exposure levels of TMT and BE activating significantly more NA neurons per tissue section than low exposure levels of the same odorants \(P < 0.05\) for each within-odor comparison; Fig. 2B). No significant interactions were observed between odor intensity level and odor type. Within subjects, there was a significant effect of rostrocaudal level (cVLM, mVLM, rVLM) \(F(2,76) = 12.8, P < 0.0001\) and a significant interaction between odor intensity and VLM rostrocaudal level on NA neuron activation \(F(2,76) = 3.4, P = 0.04\). There was no significant interaction between odor type and rostrocaudal level. Activation of NA neurons in the mVLM was significantly greater after high TMT exposure compared with high BE exposure, whereas neural activation in the cVLM and rVLM was similar after high TMT and high BE exposure (Fig. 2B). Significantly more NA neurons in the cVLM and mVLM were activated after low TMT exposure compared with low BE exposure, whereas both odorants activated similarly low numbers of NA neurons in the rVLM in the low-exposure paradigm (Fig. 2B). Figure 3 shows representative photomicrographs of c-Fos activation within the mVLM in rats after exposure to low (Fig. 3B) or high levels of TMT (Fig. 3E).

**Activation of DbH-positive NA neurons in LC (A6 cell group).** Two-way ANOVA revealed significant main effects between subjects of odor intensity (low vs. high) \(F(1,21) = 91.5, P < 0.0001\) and odor type (BE vs. TMT) \(F(1,21) = 32.0, P < 0.0001\) on c-Fos expression by NA neurons within the LC, with high exposure levels of TMT and BE activating significantly more NA neurons per tissue section than low exposure levels of the same odorants \(P < 0.05\) for each within-odor comparison. There also was a significant interaction between odor intensity and odor type on LC c-Fos expression \(F(1,21) = 17.3, P = 0.0005\). Significantly more NA cells were activated in rats after TMT compared with activation after BE at both high and low exposure levels \(P < 0.05\) for each comparison; Fig. 4). Figure 3 shows representative photomicrographs of c-Fos activation within the LC in rats after exposure to low (Fig. 3C) or high levels of TMT (Fig. 4F).

**Activation of CRH-positive PVN neurons.** As expected, similar numbers of CRH-immunopositive neurons were counted within the medial parvocellular PVN in rats represent-

Fig. 3. Representative photomicrographs depicting dual-immunoperoxidase labeling for c-Fos (black nuclear label) and dopamine β-hydroxylase (DbH; brown cytoplasmic label) within the mNST (A and D), mVLM (B and E), and LC (C and F) in rats after exposure to TMT at low levels (A–C) or at high levels (D–F). Arrows indicate some of the activated (i.e., c-Fos positive) NA neurons visible in each photomicrograph. Activation in each brain stem region is greater after high TMT exposure compared with low TMT exposure (see Figs. 2 and 4 for quantitative data).
ing each of the four odor exposure groups [low BE, 543 ± 32 (n = 7); low TMT, 527 ± 63 (n = 7); high BE, 480 ± 34 (n = 11); high TMT, 598 ± 60 (n = 9)]. Two-way ANOVA revealed significant main effects between subjects of odor intensity (low vs. high) [F(1,17) = 188.5, P < 0.0001] and odor type (BE vs. TMT) [F(1,17) = 25.3, P = 0.0001] and a significant interaction between odor intensity and type in the proportion of CRH neurons activated to express c-Fos [F(1,17) = 17.7, P = 0.0007]. High BE and high TMT exposure levels activated significantly greater proportions of CRH neurons compared with activation after low exposure levels of the same odorants (P < 0.05 for each within-odor comparison; Fig. 5). Relatively few c-Fos-positive neurons were observed within the medial parvocellular PVN in rats after exposure to low levels of BE or TMT, and there was no significant difference between these groups in the proportion of CRH neurons expressing c-Fos (Fig. 5). Exposure to high levels of TMT produced robust c-Fos expression within CRH-positive PVN neurons that was significantly greater than that in all other treatment groups and more than double the CRH activation seen in rats after high BE exposure (P < 0.05; Fig. 5). Figure 6 shows representative photomicrographs of c-Fos expression by CRH-positive PVN neurons in rats after exposure to low or high levels of BE or TMT.

**Activation of c-Fos in other brain regions.** Consistent with other reports of central c-Fos expression in rats exposed to predator-related olfactory cues (4, 6, 9, 10, 12), TMT exposure increased c-Fos immunolabeling in many brain regions beyond those subjected to quantitative analysis in the present study (data not shown). These additional regions included the interpeduncular nucleus, dorsal and ventral premammillary nuclei of the hypothalamus, ventromedial hypothalamus, paraventricular thalamus, lateral septum, median preoptic area, medial amygdala, fusiform and oval subnuclei of the bed nucleus of the stria terminalis, and olfactory cortex (i.e., piriform and dorsal endopiriform). Observed patterns of c-Fos immunolabeling generally were consistent with a recent report examining central patterns of increased c-fos mRNA expression in rats killed immediately after 30-min exposure to TMT (6).

**Tracer injection sites and distribution of retrograde labeling.** Analysis of tracer injection sites revealed that 28 rats had at least one injection accurately targeted to the center of the CeA (low BE, n = 7; high BE, n = 8; low TMT, n = 7; high TMT, n = 6). FG produced the most effective retrograde labeling in the majority of rats. To varying degrees, amygdalar regions adjacent to the CeA (i.e., the basolateral and medial nuclei) were included within the outer boundaries of CeA-centered tracer injection sites. Few or no retrogradely labeled neurons were present within the NST or VLM in rats with tracer injection sites that excluded the CeA but included adjacent regions, such as the medial or basolateral amygdala. Conversely, moderate LC neural labeling was consistently observed in cases with both accurate and inaccurate CeA injection sites. These observations are consistent with previous reports that extra-CeA regions do not receive direct neural input from the NST or VLM (28, 33, 50, 51) and support the view that neural tracer both within the CeA and within adjacent amygdalar regions contributed to retrograde LC labeling.

TMT and BE exposure activated c-Fos expression within the lateral subregion of the CeA, with particularly robust activation observed after the high TMT exposure condition (Fig. 7). Injection of FG or CTb neural tracer into this area produced retrogradely labeled neurons within the NST, VLM, and LC in all 28 cases with CeA-centered tracer injection sites, although the absolute number of labeled neurons in each brain stem region varied among cases (see Table 1). Robust retrograde labeling also was observed in lateral and medial subdivisions of the pontine PBN, although double-labeled (i.e., c-Fos and tracer positive) neurons were present only within the lateral PBN (described further in Activation of brain stem neurons projecting to CeA). Retrogradely labeled neurons were present bilaterally in all four brain stem regions, with an ipsilateral predominance of labeling relative to the side of tracer injection. No clear trends in the proportion of tracer-labeled NST or VLM neurons activated to express c-Fos were observed at any of the other brain regions considered in this study.
different rostrocaudal levels, so NST and VLM cell counts were collapsed within each experimental case.

**Activation of brain stem neurons projecting to CeA.** Three-way repeated-measures ANOVA revealed significant main effects between subjects of odor intensity (low vs. high) \( [F(1,27) = 155.6, P < 0.0001] \) and odor type (BE vs. TMT) \( [F(1,27) = 61.8, P < 0.0001] \) on activation of retrogradely labeled brain stem neurons but no significant interaction between odor intensity and type. Within subjects, there was a significant main effect of brain region (NST, VLM, LC, or PBN) \( [F(3,84) = 64.1, P < 0.0001] \) and significant interactions between odor intensity and region \( [F(3,84) = 19.2, P < 0.0001] \) and between odor type and region \( [F(3,84) = 9.9, P < 0.0001] \) on the proportion of retrogradely labeled brain stem neurons activated to express c-Fos. There also was a significant three-way interaction among odor intensity, odor type, and brain region \( [F(3,84) = 7.5, P = 0.0002] \). Significantly larger proportions of retrogradely labeled NST, VLM, and LC neurons were activated in rats exposed to high levels of BE or TMT compared with activation in rats exposed to low levels of the same odorants \( (P < 0.05 \) for each comparison; Fig. 8). Low TMT exposure activated a significantly larger proportion of retrogradely labeled neurons compared with low BE exposure in all brain stem regions \( (P < 0.05 \) for each comparison; Fig. 8). High TMT exposure activated a significantly larger proportion of NST and lateral PBN neurons compared with high BE exposure.

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**Fig. 6.** Representative photomicrographs depicting dual-immunolabeling for c-Fos (black nuclear label) and CRH (brown cytoplasmic label) within the medial parvocellular PVN in rats after exposure to BE at low (A) or high levels (B) or after exposure to TMT at low (C) or high levels (D). Activation of CRH-positive neurons is greatest after exposure to high TMT levels compared with all other conditions (see Fig. 5 for quantitative data).

**Fig. 7.** Photomicrographs depicting dual-immunoperoxidase labeling for c-Fos (black nuclear label) and CRH (brown label) within medial and lateral subregions of the central nucleus of the amygdala (CeA) in rats after exposure to BE at low (A) or high levels (B) or after exposure to TMT at low (C) or high levels (D). c-Fos activation appears greatest within the lateral CeA after exposure to high levels of TMT compared with other treatment conditions.
exposure ($P < 0.05$ for each comparison; Fig. 8), whereas activation of retrogradely labeled neurons within the VLM and LC was not significantly different between rats exposed to high levels of TMT or BE. Figure 9 shows representative photomicrographs of activated, CeA-projecting neurons located in the NST and LC in rats after exposure to low (Fig. 9, A and B) or high levels of TMT (Fig. 9, C and D).

**DISCUSSION**

Several research groups have reported that exposure to TMT, a volatile chemical component of fox feces, elicits innate behavioral responses in rats that have been described as indicative of aversion, fear, and/or anxiety. These responses include an increased incidence of unconditioned freezing and avoidance behaviors and a reduced incidence of nondefensive behaviors such as feeding, grooming, and exploration (11, 26, 44, 45). Exposure to TMT activates the hypothalamic-pituitary-adrenal (HPA) axis in rats, as evidenced by significantly increased serum levels of adrenocorticotropic hormone and corticosterone (6, 27, 43). The present study is the first to demonstrate that TMT can serve as the unconditioned stimulus for formation of CFA and the first to report anatomical data regarding the chemical phenotypes and projection targets of central neurons activated by this demonstrably aversive odorant.

The low-exposure condition provides a useful comparison for the effects of the high-exposure condition within each odorant type (i.e., TMT and BE) with the temporal features of animal transport, handling, novel environment, cage switching, and odor stimulus novelty held constant, thereby allowing us to isolate the effect of stimulus intensity within each odorant type. Our anatomical data are generally consistent with a recent report of TMT dose-related c-fos mRNA induction in widespread brain regions (6) and extend those results by reporting the chemical phenotype and axonal targets of a subset of the activated neurons. Our results indicate that TMT exposure activates CRH neurons at the apex of the HPA axis in a concentration-dependent manner, elicits a unique activation profile of brain stem NA cell populations, and activates medullary and pontine neurons that provide direct input to the CeA.

The high TMT exposure condition was associated with higher recruitment of these neurons compared with the low TMT exposure condition. A similar effect of odor intensity was observed in rats exposed to the novel and apparently nonaversive BE odor such that high exposure levels were associated with higher proportions of c-Fos activation in the phenotypically identified neurons of interest. Indeed, many of the brain regions activated in rats after TMT exposure were similar to those activated in rats after BE exposure. Areas in which activation was similar presumably reflect the responsiveness of those areas to interacting features of odor stimulus novelty and intensity that occur regardless of whether or not the stimulus is demonstrably aversive. Despite these similarities, there also were significant differences in c-Fos immunolabeling in rats exposed to TMT or BE. These differences included statistically greater activation of noradrenergic neurons in the LC and certain subregions of the NST and VLM, twice as much activation of CRH neurons in the PVN, and greater activation of CeA-projecting neurons in the NST and lateral PBN in rats exposed to high TMT levels compared with activation in rats exposed to high BE levels.

The number of volatile TMT molecules to which rats were exposed in the high- and low-exposure conditions in this study can only be estimated from those reported in a previous study (16) performed in a different exposure environment. It is important to point out that the number of volatile TMT and BE molecules to which rats were exposed may differ significantly, and it remains possible that a higher level of BE exposure might support conditioned avoidance behavior and/or produce increased c-Fos immunolabeling such that the significant differences observed in the present study are eliminated. In this regard, it will be of interest to confirm our prediction that the low TMT exposure condition used in this study is unable to support conditioned flavor avoidance, because low TMT levels produced significantly less neural activation than high BE levels in each potentially relevant brain region examined.

**Odor Exposure Paradigm**

The experimental testing environment appears to play a significant role in shaping behavioral and physiological re-

**Table 1. Retrograde labeling by brain stem region**

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>NST (μm²)</th>
<th>VLM (μm²)</th>
<th>LC (μm²)</th>
<th>Lateral PBN (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low BE (5)</td>
<td>80.3±11.6</td>
<td>52.1±9.3</td>
<td>68.4±13.7</td>
<td>118.2±10.5 (6)</td>
</tr>
<tr>
<td>Low TMT (5)</td>
<td>71.1±8.7</td>
<td>55.4±7.5</td>
<td>73.1±10.7</td>
<td>122.5±13.6 (5)</td>
</tr>
<tr>
<td>High TMT (6)</td>
<td>76.4±15.8</td>
<td>57.4±11.9</td>
<td>58.0±9.2</td>
<td>126.8±28.6 (4)</td>
</tr>
</tbody>
</table>

Values are total counts (group means ± SE) of retrogradely labeled neurons in 28 rats that received stereotaxic tracer injections into the center of the amygdala (CeA) 7–10 days before acute odor exposure. As expected, there were no significant group differences in the number of retrogradely labeled neurons within any brain stem region. *Group sizes (n) for lateral parabrachial nucleus (PBN) counts are less than for other regions because of loss of rostral pontine tissue sections in some cases. NST, nucleus of the solitary tract; VLM, ventrolateral medulla; LC, locus ceruleus.

**Odor-Induced Activation of CeA Afferents**

Fig. 8. Odor-induced activation of retrogradely labeled, CeA-projecting brain stem neurons. Bars represent the proportion (%) of retrogradely labeled neurons in each region (i.e., NST, VLM, LC, lateral PBN) activated to express c-Fos in each treatment group. For each region, bars with different letters are significantly different ($^{a,b,p} < 0.05$). Group sizes are indicated in legend. Group sizes for PBN labeling differ because of loss of sections in some animals at the pontine sectioning interface: low BE, $n = 6$; low TMT, $n = 6$; high BE, $n = 6$; and high TMT, $n = 4$. 

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sponses to TMT. Rats in our study were not acclimated to the odor exposure environment because previous work indicated that rats are less behaviorally responsive to TMT when it is presented in a familiar environmental context (26). Behavioral responses to aversive odors also may depend on the size of the testing chamber and the corresponding opportunity for the rat to “hide” or otherwise withdraw from the odor source. Several studies report robust freezing responses when rats are exposed to TMT in a small, enclosed environment (11, 16, 44, 45), as used in the present study. Other investigators using relatively large testing chambers have failed to observe unconditioned freezing responses in rats exposed to TMT (23, 27; but see Ref. 26) and also have failed to observe conditioned freezing or contact avoidance responses (2, 23). The design of the present study incorporated a novel environment and a relatively small exposure chamber to increase the likelihood of significant behavioral and physiological responses to TMT.

Aversive Nature of TMT

The finding that TMT supported the formation of a relatively strong CFA in the present study supports the view that exposure to TMT under these conditions is aversive to rats; however, the specific properties of TMT that make it aversive cannot be identified on the basis of these data. Previous reports have described TMT as a predator odor that evokes unconditioned fear and/or anxiety responses in rats or have alternatively characterized it as a repugnant odor that is aversive to rats but not fear inducing. The ability of TMT to support flavor avoidance learning in the present study could be interpreted as evidence that supports both views. Conditioned avoidance behavior could indicate that TMT generates a state of nausea or malaise. However, conditioned avoidance also could indicate that TMT generates an emotional state of fear or anxiety, and these interpretations are not mutually exclusive. Chemical agents and treatment conditions often are defined as malaise-inducing in rats on the basis of their ability to support conditioned taste aversion (CTA) (35, 40, 47, 49). CTA is operationally defined as conditioned rejection reactions (i.e., gapes, chin rubs, expelling the tastant) during intraoral infusion of a nausea-paired substance. Stimuli that support CTA learning also support CFA, but the converse is not always true (29). Further studies are necessary to determine whether TMT supports CTA in addition to the CFA demonstrated in the present study. This is of interest because other chemical agents such as cholecystokinin and lipopolysaccharide that support both CTA and CFA in rats have been reported to induce both malaise and anxiety in humans and experimental animals (14, 17). In this regard, anxiety in humans often is associated with feelings of malaise and nausea, and vice versa, suggesting a functional overlap of central neural pathways recruited by anxiogenic and nauseogenic stimuli (1, 21).

Comparison of Neural Activation After TMT or BE

The control odor (BE) used in this study was selected on the basis of its novelty to rats, its subjectively similar aromatic potency to TMT (as judged by human observers), and our unpublished observations that rats do not avoid drinking water that contains 0.5% BE. In the present study, the lack of CFA in rats after BE exposure indicates that BE is not aversive, at least not in a manner that can be detected in the sensitive two-bottle choice test. Nevertheless, exposure of odor-naive rats to high levels of BE produced a statistically significant increase in the proportion of CRH neurons activated to express c-Fos compared with activation in rats after exposure to low levels of BE or low levels of TMT. It is important to note, however, that activation of CRH neurons within the PVN in rats after high BE exposure was less than one-half of the CRH activation observed in rats after high TMT exposure, evidence that TMT
elicited a significantly greater response of neurons at the apex of the HPA axis at these odor exposure levels. The uniquely robust CRH activation observed in rats after high TMT exposure is particularly noteworthy when one considers that high BE and high TMT exposures activated similar proportions of NA neurons at certain rostrocaudal levels of the NST and VLM (Fig. 2). As discussed, we did not determine whether lower exposure levels of TMT (i.e., conducted near a fume hood as in the c-Fos studies) are sufficient to support CFA; thus it remains unclear whether odor-induced activation of CRH neurons is correlated with the ability of the odor to support CFA.

Although NA neurons at some rostrocaudal levels of the NST and VLM were activated to a similar extent in rats after exposure to high BE or high TMT levels, TMT recruited slightly, but significantly, larger numbers of NA cells within the rNST and mVLM (Fig. 2) and activated ~60% more NA neurons in the LC (Fig. 4) compared with activation after BE. The differential responses of these particular NA neurons to BE and TMT presumably reflects the different sensory qualities of the two odors, their perceived significance to the animal, and/or the animal’s corresponding behavioral/physiological responses. The greater TMT-induced activation of LC neurons is of special interest because the LC provides widespread input to many brain regions that do not receive direct input from either the NST or VLM but have been implicated in defensive behavior. Several additional cortical and subcortical regions showed increased c-Fos expression after TMT exposure in the present study, although we have not yet quantified those data or compared them statistically with neural activation after control odor exposure.

Odor-Induced Activation of Viscerosensory Pathways

Odor-induced recruitment of medullary and pontine NA cell groups could occur via descending neural circuits arising in the limbic forebrain after olfactory stimulation and/or from viscerosensory feedback subsequent to odor-induced physiological and behavioral responses. Olfactory information is sent directly to the amygdala, including the CeA (42), which has well-characterized descending projections to hindbrain NA cell groups. Indeed, results from several studies suggest that interoceptive stressors may recruit medullary NA neurons, in part, by inhibiting descending GABA-ergic pathways from the medial CeA (7, 20, 34). It also is possible that odor-induced activation of hindbrain NA cell groups occurs subsequent to interoceptive feedback signaling to the brain along pathways that route through viscerosensory subregions of the NST, VLM, and PBN en route to the hypothalamus and limbic forebrain.

TMT-Induced Activation of Inputs to CeA

There is growing evidence that viscerosensory inputs to the amygdala play a critical role in integrating adaptive behavioral and physiological responses to stress and are important for conditioned learning and the encoding of memories for emotionally provocative stimuli (5, 22, 48). The present study is the first to demonstrate that amygdala-projecting neurons in the caudal medulla and pons are activated by a demonstrably aversive odor. Our findings are consistent with a recent report of c-fos mRNA induction in rats after TMT exposure, in which activation of cells in the CeA was highly correlated with activation of cells in the lateral PBN and the NST, among other regions (6). Together, these findings support the view that TMT exposure recruits visceral sensory pathways in a manner similar to recruitment observed after other types of interoceptive challenge (6, 28). We did not determine the chemical phenotypes of retrogradely labeled neurons in this study, but previous reports have indicated that medullary and pontine inputs to the CeA feature NA neurons in the NST, VLM, and LC and calcitonin gene-related peptide (CGRP)-positive neurons in the lateral PBN (28, 33, 39, 50, 51). Additional direct inputs to the CeA arise from glucagon-like peptide 1 neurons in the caudal NST (13, 19, 25). Interestingly, a significantly greater proportion of afferent inputs to the CeA arising in the NST, VLM, and LC were activated after low TMT exposure compared with low BE exposure. Furthermore, in the high odor exposure paradigm, a differential effect of TMT-induced neural activation was observed only within CeA inputs arising in the NST and PBN. The additional CeA-projecting NST and PBN neurons recruited in rats after high TMT exposure may include glucagon-like peptide-1-positive NST neurons and CGRP-positive lateral PBN neurons that provide direct input to the CeA. In this regard, the TMT-specific activation of c-Fos expression in the external lateral PBN is consistent with the observed CFA response to TMT, because formation of both CFA and CTA requires the PBN, the CeA, and the connections between them (15, 18, 35, 49). Our preliminary observations indicate that many of the lateral PBN neurons activated in rats after TMT exposure are CGRP positive and that c-Fos-positive CeA neurons in the same rats lie within the lateral CeA subregion region that is targeted by CGRP-positive fibers (data not shown).

In summary, we conclude that TMT is an aversive odorant in rats on the basis of its ability to serve as the unconditioned stimulus for a significant conditioned flavor avoidance. Acute exposure to levels of TMT that support conditioned avoidance produces a relatively distinct pattern of neural activation in identified hypothalamic CRH neurons, brain stem NA neurons, and medullary and pontine neurons that provide direct afferent input to the CeA. We propose that the ability of TMT to support conditioned avoidance behavior is directly related to odor-induced recruitment of ascending viscerosensory projection pathways from the pons and caudal medulla to the amygdala. The results of this study provide a general framework with which to further probe neural substrates that underlie specific aspects of behavioral and physiological responses to aversive stimuli.

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