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# fMRI fingerprint of unconditioned fear-like behavior in rats exposed to trimethylthiazoline

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**Abstract**

Unconditioned fear plays an important yet poorly understood role in anxiety disorders, and only few neuroimaging studies have focused on evaluating the underlying neuronal mechanisms. In rodents the predator odor trimethylthiazoline (TMT), a synthetic component of fox feces, is commonly used to induce states of unconditioned fear. In this study, arterial spin labeling-based functional magnetic resonance imaging (fMRI) was applied to detect TMT-induced regional modulations of neuronal activity in Wistar rats. During TMT exposure the rats displayed increased freezing behavior and reduced exploration in the odor-associated area. Neuronal activity was selectively increased in the dorsal periaqueductal gray, superior colliculus and medial thalamus and reduced in the median raphe, locus coeruleus, nucleus accumbens shell, ventral tegmental area, ventral pallidum and entorhinal piriform cortex. This fMRI fingerprint involving distinct neuronal pathways was used to describe a schematic model of fear processing. Key brain areas known to underlie fear and anxiety-related autonomic and behavioral responses as well as centers of motivational processing were identified as being part of this functional circuitry of innate fear. Thus, preclinical fMRI studies based on unconditioned fear methods may provide a valuable translational approach to better characterize etiological and pathological processes underlying anxiety disorders.


## 1. Introduction

Fear and defensive behaviors are adaptive responses that help an individual survive life-threatening situations. These responses include specific physiological and behavioral reactions induced by both learned (conditioned) danger-

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predicting stimuli and unlearned (unconditioned) fear-inducing stimuli (LeDoux, 2000; Misslin, 2003). It is now thought that innate predispositions and not environmental experiences are significant factors that underlie certain anxiety disorders such as phobias (Kendler et al., 2002). Similarly, spontaneous panic attacks may be an emotion of unconditioned fear and can be distinguished from generalized anxiety disorder (Battaglia and Ogliari, 2005). In this context, predator odors, such as cat and fox odor, which indicate immediate danger in prey animals and provoke defensive and escape behavior, have been used to induce states of unconditioned fear in rodents (Apfelbach et al., 2005; Blanchard et al., 2005). The main behavioral responses provoked by predator odor are avoidance, risk assessment and defensive immobility that are initiated to avoid detection by the predator (Misslin, 2003; Takahashi et al., 2005). Recently, the predator-associated olfactory cue trimethylthiazoline (TMT), a synthetically reproduced component of fox feces, has been used to induce innate unconditioned fear in rodents (Fendt et al., 2005; Vernet-Maury et al., 1984). TMT is a pure synthetic molecule that can be precisely dosed, thus ensuring well-controlled odor concentrations for a better standardization of experiments.

In the present study continuous arterial spin labeling (CASL)-based fMRI in anesthetized rats was applied to evaluate TMT-induced changes in blood perfusion. The concept of fMRI postulates that locally measurable changes in blood perfusion mirror alterations of neuronal activity in distinct brain areas (Fox and Raichle, 1986). Both basal brain activity and the effect of drugs can be reliably measured by CASL-based fMRI in rats (Bruns et al., 2009; Nordquist et al., 2008; Risterucci et al., 2005). Moreover, CASL-based fMRI does not require the use of exogenous contrast agents and provides a quantitative and temporally more stable imaging modality than blood-oxygenation-level-dependent imaging (Wang et al., 2003). Anesthesia of animals during fMRI image acquisition is often discussed as it has an effect on neuronal activity *per se*. Without anesthesia, however, accurate images are difficult to obtain because motion artifacts are present unless animals are immobilized by paralysis or acclimatization to a tight mechanical constraint (Lahti et al., 1998) which are likely to have their own ramifications on experimental results. Alternatively, conducting animal fMRI studies under well-chosen isoflurane anesthesia has been shown to be a reliable approach in order to obtain robust and meaningful data on brain neurocircuitry (Kalisch et al., 2004; Nordquist et al., 2008; Rausch et al., 2005; Risterucci et al., 2005). The current study was designed to assess blood perfusion changes associated with the anxiogenic effects of TMT as a means to identify brain areas involved in processing of unconditioned fear. As TMT is suspected to comprise acrid properties that could interfere with the anxiogenic effect of the odor (Fendt and Endres, 2008), we compared the TMT-induced neuronal fingerprint to changes identified after exposure to butyric acid (BA), an acrid substance that induces avoidance behavior in rodents but no anxiety (Endres and Fendt, 2009; Wallace and Rosen, 2000). In addition, the behavioral alterations triggered by exposure to TMT and BA were analyzed to confirm the different properties ascribed to both odorants. Altogether, these data were used to delineate a possible functional neuronal network that is involved in the response to unconditioned fear.

## 2. Experimental procedures

### 2.1. Animals

Animal procedures were conducted in strict adherence to the Swiss federal regulations on animal protection with the explicit approval of the local veterinary authority and to the rules of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Male Wistar rats (Harlan, Netherlands) weighing 250 g at the beginning of the experiment were housed individually and maintained in temperature-controlled conditions with a 12 h light/dark cycle (6 a.m.–6 p.m., lights on). Food and water were provided *ad libitum*.

### 2.2. Odor exposure

Odor exposure was conducted in a transparent Plexiglas box (41×41×28 cm; wood shavings for bedding) over 20 min. TMT (100 μl; 2,3,5-trimethyl-3-thiazoline; Phero Tech, Delta, Canada), BA (300 μl; Sigma-Aldrich) or water (100 or 300 μl) as the odor-free control were applied on a filter paper (3×3 cm) and presented on a small glass dish inside the Plexiglas box. The dose of TMT was chosen in accordance with an earlier fMRI study in rats showing significant changes in brain activity after TMT exposure (Chen et al., 2007). Furthermore, the dose of BA was three times that of TMT as it is three times less volatile (Hotsenpiller and Williams, 1997). Rats were tested in "odor" groups: i.e., animals were first exposed to water then either TMT or BA. This allowed for a thorough cleaning and ventilation of the exposure room and avoided potential cross-contamination between the groups. The exposure box was thoroughly cleaned after each rat using an ethanol-propanol-based disinfectant and the bedding was exchanged. Behavioral assessment during odor exposure and fMRI acquisition after odor exposure was performed in different sets of animals.

### 2.3. Behavioral assessment

To assess the behavioral response to odor exposure, animals were introduced into the Plexiglas box in the corner opposite to the one where the glass dish containing the odor-moistened filter paper was placed and behavior was scored manually over 20 min. The following parameters were measured: time spent in the quadrant in which the moistened filter paper was located (proximal zone); number of entries and latency to the first entry into the proximal zone; duration, latency and number of contacts with the filter paper; number of freezing episodes (cessation of movements except of those necessary for breathing). The effects of TMT and BA as compared to water were assessed in two separate experiments (TMT vs. water, n=8 each; BA vs. water, n=10 each).

### 2.4. Functional magnetic resonance imaging (fMRI)

On the day of the fMRI study, conscious animals were first exposed to either TMT, BA or water as described above. Perfusion image acquisition was then performed 30 min following odor exposure (for details see below).

Animals were anesthetized with an induction level of 4% and a maintenance level of 2–2.5% isoflurane (Abbott, Cham, Switzerland) in a mixture of oxygen (0.2 l/min) and air (1.0 l/min) administered via a face mask 10 min following the end of odor exposure. Rats were positioned in a plastic cradle in the magnet and the head immobilized

in a stereotaxic holder. Body temperature was maintained at 37 °C using a feedback-regulated electric heating blanket. Breathing rate and concentrations of inhaled and exhaled oxygen and $CO_2$ were continuously monitored on a PowerLab data acquisition system (ADInstruments, Spechbach, Germany). Images were obtained using a Bruker Biospec 4.7 T/40 cm instrument (Bruker Biospin, Ettlingen, Germany) equipped with a 12 cm actively shielded gradient set. A 7 cm diameter birdcage coil was used for radio-frequency excitation and an actively decoupled surface coil was positioned on the head of the animal for signal reception. For all images, the field-of-view was 4 cm and the slice thickness 1 mm.

A set of scout images ($T_2$-weighted RARE, repetition time (TR) = 2.7 s, echo spacing (TE) = 10.3 ms, RARE-factor 8, 128×64 matrix, 4 averages) in axial orientation was first acquired in each animal in order to locate the most rostral extension of the corpus callosum, which served as landmark for the subsequent study. A set of anatomical images ($T_2$-weighted RARE, TR/TE = 1.8 s/18.0 ms, RARE-factor 8, 256×256 matrix, 4 averages) was obtained from eight coronal slices at +2.3, +1.0, −0.3, −1.6, −2.9, −5.3, −7.8 and −10.0 mm compared to bregma (Paxinos and Watson, 1986). $T_1$-maps required to quantitate perfusion were collected using an inversion-recovery-snapshot-FLASH sequence with eight inversion times (TR/TE = 7.5 s/1.7 ms, 128×64 matrix, 8 averages) (Haase et al., 1986). 30 min after TMT exposure termination, perfusion imaging was conducted based on the CASL method (Alsop and Detre, 1996; Williams et al., 1992) with a RARE readout module (TR/TE = 3 s/5.5 ms, RARE-factor = 32, 128×64 matrix, 2 averages, 2.5 s labeling pulse) (Bruns et al., 2009). The eight image planes for each time-point were acquired in 4 min. Three sets of images of basal perfusion, corresponding to a 12-min period, were recorded to assess within-subject reproducibility, and were averaged before entering the subsequent analyses to improve the signal-to-noise ratio. Effects of TMT and BA as compared to water were assessed in two separate experiments (TMT vs. water, n = 8 each; BA vs. water, n = 8 each).

## 2.5. Imaging data analysis

Magnetic resonance images were processed and analyzed using in-house software written in *IDL* (RSI, Boulder, CO) and *MATLAB* (The MathWorks Inc., Natick, MA). Anatomical brain images of each animal were co-registered to a previously obtained template with an associated in-house digital atlas. The atlas was generated based on an individual template animal, in which various brain areas of potential interest were delineated by manually overlaying and adapting the contours of the rat brain atlas by Paxinos and Watson (1986). A total of 35 brain areas were defined (for a detailed list see Table 1). Co-registration was carried out automatically using the open-source software SPM5 (Wellcome Trust Centre for Neuroimaging, London, UK) and comprised a 12-parameter affine transform as well as a nonlinear normalization to the template. The same transform was applied to all functional images. $T_1$-maps were calculated on a pixel-by-pixel basis by applying a three-parameter exponential fitting algorithm (Deichmann et al., 1999). Absolute perfusion maps were obtained using the CASL-images and the quantitative $T_1$-map as described elsewhere (Alsop and Detre, 1996; Bruns et al., 2009). In order to compensate for possible systemic hemodynamic changes, perfusion was normalized to the mean of each slice and expressed as the percentage derivation of the mean. For each animal, the mean normalized perfusion value was calculated within the various brain areas of interest. For graphical representation, the perfusion images of all animals of a specific treatment were superposed and averaged in order to obtain representative images.

## 2.6. Statistical analysis

Behavioral data from rats exposed to TMT, BA, or water were analyzed using Student's *t*-test (JMP 6 software, SAS Institute Inc., NC). All fMRI data were compiled in a Microsoft Excel datasheet (Excel 2002, Microsoft, Redmond, WA). fMRI-based regional blood perfusion was compared between TMT- or BA- and water-exposed rats using a two-way ANOVA with factor treatment and brain areas followed by unpaired *t*-test separately for each brain area (JMP 6 software, SAS Institute Inc., NC).

Given the exploratory character of the present study with the goal of identifying the area(s) being potentially most relevant to the mechanisms in question, we preferred accepting reasonably small numbers of false positives over large numbers of false negatives. Hence, instead of using the low-powered approaches for controlling the family wise error rate (e.g., Bonferroni, Šidàk, Holm, Hochberg) or multivariate techniques (which would have required an unacceptably large number of subjects), we applied the standard significance criterion of $p<0.05$ with no correction for multiple testing, and estimated the false discovery rate (FDR; Storey and Tibshirani, 2003). Areas meeting this plain significance criterion are reported as findings of primary interest, keeping in mind that they are expected to include a defined small number of false positives ($n_{FP}$). In the present study, the expected number of false positives in no case exceeded $n_{FP} = 2$ areas, with an upper 90% confidence bound of $n_{FP} = 4$ (confidence intervals estimated via bootstrapping; (Storey, 2002)). Results presented herein are expressed as mean ± SEM except where indicated otherwise.

**Table 1** Brain areas examined by fMRI.

| | |
|---|---|
| 1 | Dorsal medial prefrontal cortex |
| 2 | Ventral medial prefrontal cortex |
| 3 | Orbitofrontal cortex |
| 4 | Sensory cortex 1 |
| 5 | Sensory cortex 2 |
| 6 | Motorcortex 1 |
| 7 | Entorhinal piriform cortex |
| 8 | Perirhinal cortex |
| 9 | Dorsal striatum |
| 10 | Nucleus accumbens core |
| 11 | Nucleus accumbens shell |
| 12 | Ventral pallidum |
| 13 | Bed nucleus of stria terminalis |
| 14 | Ventral tegmental area |
| 15 | Substantia nigra |
| 16 | Locus coeruleus |
| 17 | Thalamus |
| 18 | Lateral thalamus |
| 19 | Medial thalamus: mediodorsal part |
| 20 | Medial thalamus: other nuclei |
| 21 | Basolateral amygdala |
| 22 | Dorsal hippocampus: CA |
| 23 | Fimbria |
| 24 | Ventral hippocampus |
| 25 | Dorsal hippocampus: posterior |
| 26 | Septum |
| 27 | Dorsal periaqueductal gray |
| 28 | Ventral periaqueductal gray |
| 29 | Dorsal raphe nucleus |
| 30 | Median raphe nucleus |
| 31 | Superior colliculus |
| 32 | Inferior colliculus |
| 33 | Paraventricular hypothalamus |
| 34 | Lateral hypothalamus |
| 35 | Median hypothalamus |

## 3. Results

### 3.1. Odor exposure-associated behavioral alterations

During the 20-min observation period, rats exposed to TMT spent significantly less time investigating the filter paper ($p<0.001$) and had a significantly reduced number of contacts ($p<0.001$) and an increased latency to the first contact ($p<0.01$, Table 2). Furthermore, TMT-exposed rats spent significantly less time in ($p<0.01$) and showed less entries into ($p<0.01$) the proximal zone in which the TMT-moistened filter paper was placed as compared to water-exposed rats. Latency to the first entry into the proximal zone also tended to increase as compared to water-exposed rats ($p=0.06$). Additionally, the number of freezing was significantly increased ($p<0.05$) in TMT-exposed rats when compared to water-exposed animals (Table 2).

Exposure to BA resulted in a significant decrease in time ($p<0.05$) and number ($p<0.05$) of contacts with the filter paper and in time spent in the proximal zone ($p<0.05$) when compared to water-exposure (Table 2). BA-exposed rats did not show more freezing behavior than water-exposed animals.

### 3.2. Odor exposure-associated brain activity changes

To evaluate brain activity changes induced by the exposure to the predator odor TMT or the acrid substance BA, regional blood perfusion was measured in specific brain areas 30 min after the end of the odor-exposure using fMRI. Fig. 1 shows the differences in normalized perfusion between TMT- and water-exposed animals in eight coronal brain sections. The overall ANOVA showed no significance for the factor "treatment" ($F_{1,490}=0.86$, $p=0.35$), but did so for the interaction "treatment × brain area" ($F_{34,490}=3.02$, $p<0.0001$) and, not surprisingly, for the factor "brain area" ($F_{34,490}=122$, $p<0.0001$). Changes in blood perfusion in specific brain areas were explored by post-hoc t-tests. Specifically, rats exposed to TMT exhibited a significant increase in perfusion of the dorsal PAG ($p<0.01$, Fig. 2A), superior colliculus ($p<0.05$, Fig. 2C) and the mediodorsal thalamus ($p<0.01$, Fig. 2B) as compared to rats exposed to water. Blood perfusion was significantly decreased in entorhinal piriform cortex ($p<0.01$, Fig. 2C), ventral tegmental area (VTA, $p<0.01$), shell of the nucleus accumbens ($p<0.05$), ventral pallidum ($p<0.001$, Fig. 2B) as well as in median raphe ($p<0.001$) and locus coeruleus ($p<0.05$, Fig. 2A) in animals exposed to TMT-when compared to those with water. Furthermore, a trend toward a decrease in cerebral perfusion after TMT exposure was found in the basolateral amygdala (BLA; $p=0.077$), paraventricular nucleus of the hypothalamus (PVN; $p=0.096$, Fig. 2A) and posterior dorsal hippocampus ($p=0.074$, Fig. 2C); a trend for increased perfusion was measured in the inferior colliculus ($p=0.094$, Fig. 2C). In the substantia nigra, dorsal striatum, nucleus accumbens core (Fig. 2B), dorsal raphe nucleus and ventral PAG (Fig. 2A) as well as in medial prefrontal cortex and septum, (data not shown) no significant changes in blood perfusion were detected.

The overall ANOVA comparing BA- and water-exposed animals showed a significant effect for the factor "brain area" ($F_{34,490}=65.6$, $p<0.0001$), but no significant effects for the factor "treatment" ($F_{1,490}=0.91$, $p=0.34$) and the interaction "treatment × brain area" ($F_{34,490}=0.96$, $p=0.54$), indicating that BA exposure had no significant overall or differential effect on brain activation.

To assess within-subject reproducibility, the 3 successively acquired perfusion images were used to estimate, for each treatment and each brain area, the intra-class correlation (ICC) for the average of the 3 acquisitions, based on a random-effects model (i.e., ICC(2,3) from Shrout and Fleiss, 1979). ICC values of normalized perfusion in the various brain areas (after averaging across treatments) were distributed around an overall mean of $ICC_{NrmPrf}=0.91$ (ordinary standard-deviation interval: 0.82…0.95, which is asymmetric about the mean due to the skewed distribution of correlation values). This shows that

**Table 2** Behavioral response during odor exposure.

| | TMT (n=10) | Water (n=10) | BA (n=8) | Water (n=8) |
|---|---|---|---|---|
| *Proximal zone* | | | | |
| Time in zone [s] | 19±4 *** | 322±43 | 177±31 * | 413±87 |
| Number of entries | 4±1 *** | 20±2 | 16±1 | 20±2 |
| Latency to 1st entry [s] | 181±7 [a] | 35±8 | 84±27 | 62±29 |
| *Source of odor* | | | | |
| Time of contact [s] | 3±1 *** | 197±42 | 15±5 * | 75±20 [b] |
| Number of contacts | 2±0 *** | 21±3 | 6±2 * | 15±3 |
| Latency to 1st contact [s] | 399±118 ** | 38±8 | 119±40 | 78±27 |
| *Freezing* | | | | |
| Duration of freezing [s] | 6±2 * | 0±0 | 2±1 | 0±0 |

Data are presented in mean±SEM; Student's *t*-test:
* $p<0.05$.
** $p<0.01$.
*** $p<0.001$ vs. water.
[a] $p=0.06$ vs. water.
[b] $p<0.05$ vs. water of TMT-experiment.

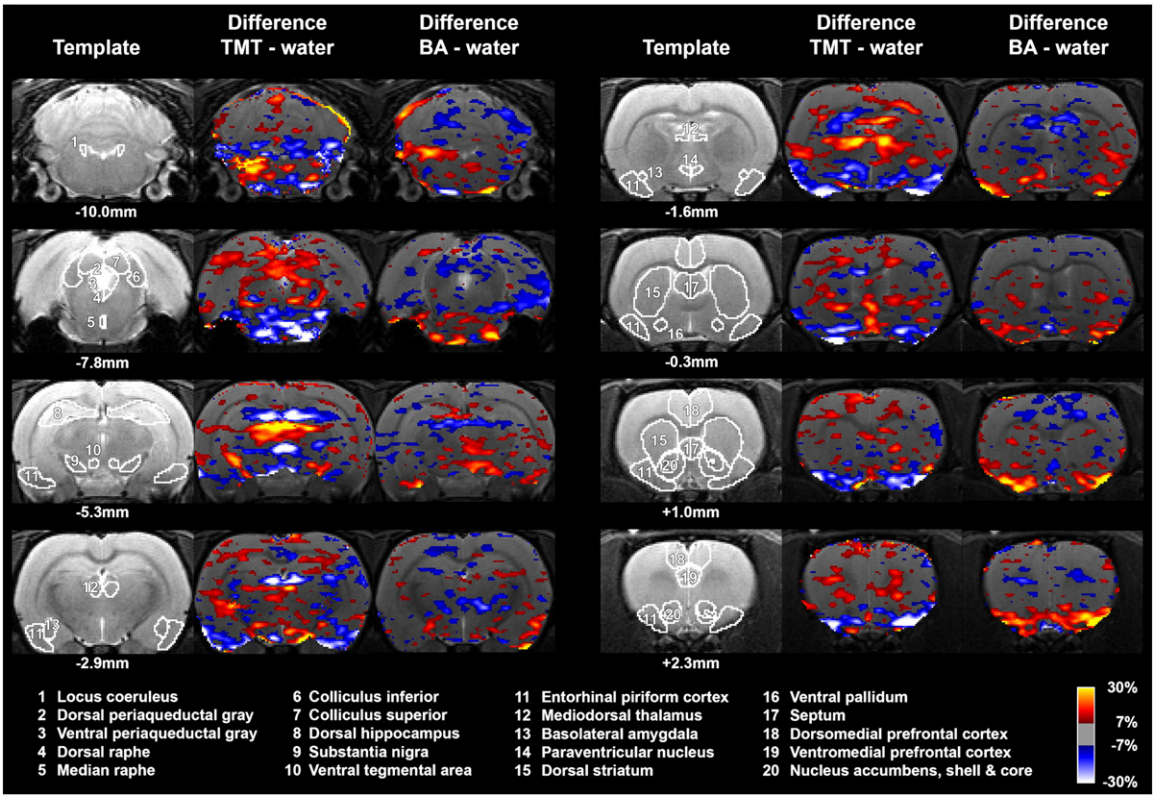


**Figure 1** TMT- and BA-induced perfusion changes in naive Wistar rats. Frontal anatomical brain images (template) with corresponding anteriorities from bregma according to the Paxinos and Watson rat brain atlas (1986) show the delineated areas of interest (1–20, see legend). Perfusion normalized to the mean of each slice (expressed as percentage) in each rat was calculated and then averaged for rats belonging to the TMT, BA or water treated groups, respectively. The color images to the right of each template show the color-coded differences in normalized perfusion between odor- (TMT or BA) and water-exposed rats at the corresponding brain anteriority. A 7% threshold was applied to the color coding which approximately reflects the noise level relevant to the group comparisons made in the present study, i.e., the (inter- plus intra-subject) variability of area-mean values.

measurement noise was only a minor source of variability. To confirm reproducibility at the treatment-group level, we compared the patterns of normalized perfusion across the various brain areas between the two control treatments (water). Consistency was reasonably high, showing a Pearson correlation coefficient of $r_{H2O/H2O}=0.91$ (95% confidence interval: 0.86…0.95).

## 4. Discussion

The current study demonstrates that exposure to the predator odor TMT selectively modifies brain activity in Wistar rats. fMRI identified significantly higher neuronal activity in the dorsal PAG, superior colliculus, and mediodorsal thalamus and lower activity in the entorhinal and piriform cortex, VTA, ventral pallidum, shell of nucleus accumbens, median raphe and locus coeruleus after TMT exposure. In contrast, exposure to the noxious agent BA did not result in any significant activity changes. On a behavioral level, rats exposed to BA displayed avoidance of the source of the odor whereas exposure to TMT resulted in increased freezing behavior, reflecting fear, as well as reduced contact to the odor and reduced exploratory behavior, thus confirming earlier findings (Rosen et al., 2006). The TMT-induced fingerprints of neuronal activities have been synthesized in a schematic model of fear processing that identifies key structures involved in the coordination of the autonomic and behavioral responses to fear-inducing stimuli and the integration of emotion-motivation (Fig. 3).

Regarding the autonomic-behavioral response associated with TMT exposure, neuronal activity in the dorsal part of the PAG, an area associated with defensive behaviors of the fear response in rodents (De Oca et al., 1998), was significantly increased after exposure to TMT. The dorsal PAG is known to be a key structure mediating context-independent unconditioned freezing (Vianna et al., 2001) and escape (Zanoveli et al., 2004) both reflecting panic-like behavior (Brandao et al., 2008). Noradrenaline was also shown to be critically involved in conditioned fear (Roozendaal et al., 2006) and to modulate PAG activity (Pelosi et al., 2009). The decreased neuronal activity observed in the locus coeruleus after TMT exposure suggests that inhibition of locus coeruleus activity might be required to run proper processing of unconditioned fear. Similarly, TMT exposure also resulted in lower activity in the median raphe. This suggests that the TMT-induced processing of unconditioned fear might inhibit median raphe activity and its control of the strongly interconnected septohippocampal system (Almada et al., 2009). The fMRI

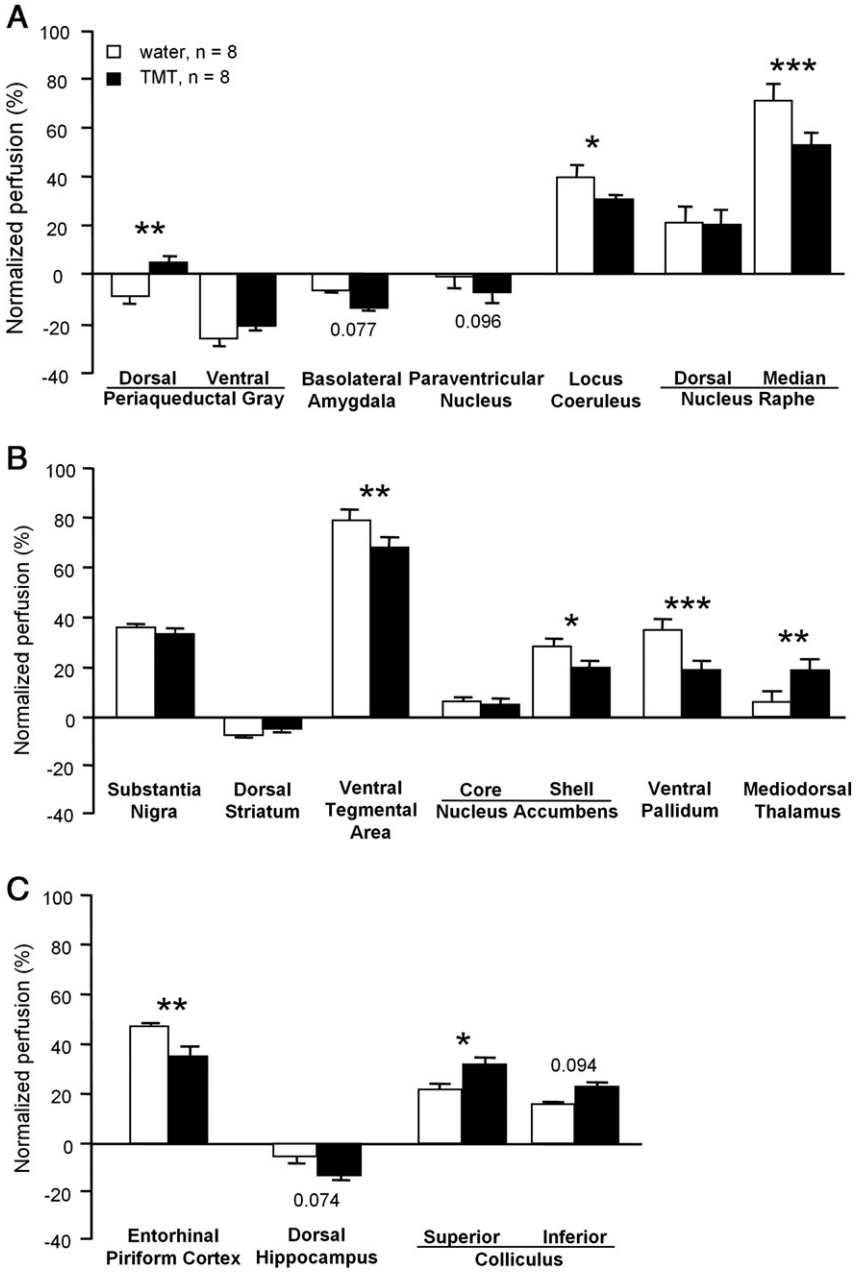


**Figure 2** Brain activity measurements in water- (open bars, n=8) and TMT- (filled bars, n=8) exposed rats. Differences in normalized perfusion (percent change from the mean slice perfusion) of brain areas involved in (A) the autonomic-behavioral response, (B) emotion-motivation integration and (C) emotion-cognition integration and processing of sensory stimuli were assessed by two-way ANOVA followed by an unpaired *t*-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

data also show a moderate activity change in the BLA. Although the amygdala is known to coordinate the autonomic-behavioral fear response (Chen et al., 2007; LeDoux, 2000), the BLA, in contrast to the medial amygdala, has not been strongly implicated in unconditioned fear (Muller and Fendt, 2006). A modest effect in the PVN was also observed in the current study. The PVN is critical for the neuroendocrine stress response regulating hypothalamus–pituitary–adrenocortical axis activity (Herman et al., 1996) and PVN c-fos activity has been shown to increase after predator-odor exposure (Day et al., 2004; Munoz-Abellan et al., 2008).

With regard to the emotion-motivation response associated with TMT exposure, perfusion changes in the VTA, nucleus accumbens shell and ventral pallidum indicate the selective involvement of the mesolimbic dopaminergic system in the TMT-induced fear response (Fig. 3). The mesolimbic dopaminergic system integrates motivational aspects of reward or aversion into fear response processing (Bressan and Crippa, 2005). In particular, the shell of the nucleus accumbens is known to mediate especially the motivational aspects of unconditioned stimuli and to influence unlearned behaviors (Bassareo and Di Chiara, 1999; Stratford and Kelley, 1999). The

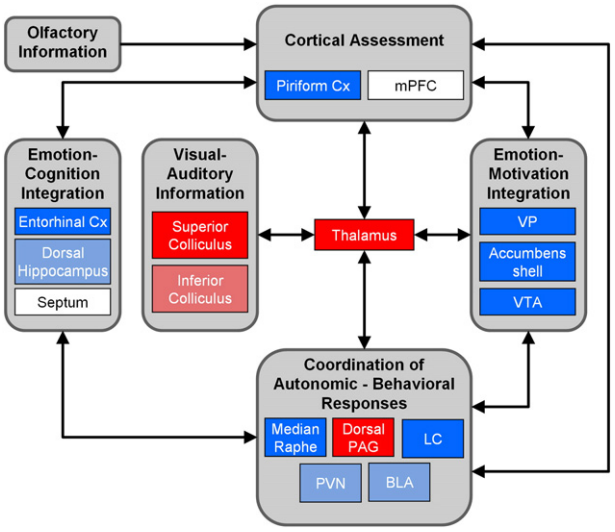


**Figure 3** Schematic representation of the functional neuronal network of brain areas involved in the response to unconditioned fear. Dark red and blue boxes respectively represent the significant increases or decreases of neuronal activity in the corresponding brain regions observed upon TMT exposure; light red and blue coloring represent trends to an increase or decrease respectively in neuronal activity. BLA, basolateral amygdala; PVN, paraventricular nucleus of the hypothalamus; LC, locus coeruleus; PAG, periaqueductal gray; VP, ventral pallidum; VTA, ventral tegmental area; mPFC, medial prefrontal cortex; Cx, cortex.

TMT-induced decrease of activity in the VTA, ventral pallidum and nucleus accumbens shell might reflect either a predator odor-associated state of aversion or recovery from an aversive situation when the animal is no longer exposed to the predator odor. The increased mediodorsal thalamus signal can be linked to activity in the mesolimbic dopaminergic structures. Indeed, the mediodorsal thalamus is a central relay connecting the ventral striatum with cortical areas to integrate motivational aspects to situation-related cortical assessment processes of exogenous and endogenous stimuli (Cardinal et al., 2002).

Exposure to BA did not lead to any activity changes in the rat brain. This finding strongly supports the relation of the TMT-induced activity pattern to processing of fear and anxiety free from activity changes related to the information processing of a noxious olfactory stimulus. Neuronal activity in the septohippocampal system was decreased and prefrontal cortical areas displayed no alterations in activity after TMT exposure (Fig. 3) suggesting that cognitive processing of contextual cues (Bast, 2007) and cortical assessment of the fear-inducing situation (Cardinal et al., 2002) play a minimum role in neuronal processing of predator odor-induced unconditioned fear. Although reduced signal intensity in prefrontal and cingulate cortex after TMT exposure was recently shown (Chen et al., 2007), comparable activation in prefrontal areas were associated with general olfactory processing (Day et al., 2004; Hebb et al., 2004). In the present study, olfactory processing of the stimulus is translated in significant activity change in the piriform cortex the primary olfactory cortex (Illig and Haberly, 2003) and the entorhinal cortex (Kerr et al., 2007). Interestingly, the high neuronal activity found after TMT exposure in superior and inferior colliculi might reflect the fast processing of visual and acoustic information in subcortical pathways (involving PAG and amygdala (Brandao et al., 1999)) that are selective for fear-relevant stimuli (LeDoux et al., 1990; Linke et al., 1999).

Anesthesia of animals following exposure to the odor may have impacted the TMT-induced activity changes. However, conducting animal fMRI studies under well-chosen isoflurane anesthesia versus awake animals is considered a highly valid approach to obtain reliable and meaningful data on brain neurocircuitry (Kalisch et al., 2004; Nordquist et al., 2008; Rausch et al., 2005; Risterucci et al., 2005). In the present study, fMRI examination was performed 30 min following odor exposure. Although we identified significant activity changes in various brain areas after TMT exposure that were not found after corresponding exposure to BA, the temporal distance between odor exposure and image acquisition and the lack of behavioral read-out at that time may weaken the association of the found neuronal activity changes with TMT-induced fear and anxiety. In support of our findings, however, different studies have demonstrated long-lasting effects of TMT exposure on anxiety up to three days after TMT exposure (Fendt et al., 2005; Hebb et al., 2003; Hebb et al., 2004). Yet, it shouldn't be assumed that neuronal processing of fear is at a stable state over the time of the experiment and thus certain highly transient effects may have eluded detection, e.g., TMT-induced increased activity in amygdala and PVN that might be dampened 30 min after exposure due to negative feedback mechanisms. Nevertheless, the identified significant activity changes describe a highly relevant network of brain areas responding to innate fear and anxiety stimuli.

Imaging studies of panic attacks in humans showed altered neuronal activation in the PAG, hypothalamus, amygdala, thalamus, superior colliculus, hippocampus, insula and parahippocampus (corresponding to the entorhinal cortex in rats) (Boshuisen et al., 2002; Fischer et al., 1998; Javanmard et al., 1999; Pfleiderer et al., 2007; Reiman et al., 1989). Furthermore, a hierarchical shift in brain activity (McNaughton and Corr, 2004) from ventromedial prefrontal cortex to PAG depending on the distance to a threat was recently shown in a fMRI study exposing healthy volunteers to a virtual predator (Mobbs et al., 2009). The strong activity increase in the dorsal PAG with minor involvement of cortical and hippocampal areas after TMT exposure discovered in the present study (Fig. 3) may reflect this unconditioned response to a close threat.

In conclusion, the current data set shows that fear-related behavior induced by TMT exposure is associated with activation change in specific areas of the rat brain, including panic- and fear response-related areas and regions mediating reward and aversion (Fig. 3). The similarity between clinical data and outcomes of the present study suggests that TMT exposure is a reliable procedure to induce unconditioned fear in rodents and that underlying neuronal circuitries can be characterized by means of fMRI.

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## Contributors

C. Risterucci, M.S. Keßler and J.-L. Moreau designed the study; S. Debilly, S. Schöppenthau, and M.S. Keßler performed the experiments; B. Künnecke, A. Bruns and T. Bielser set up the fMRI protocols and analysis programs. M.S. Keßler and C. Risterucci wrote the manuscript; A. Bruns, B. Künnecke, M. von Kienlin, J.G. Wettstein and J.-L. Moreau edited the manuscript.

## Conflict of interest

The authors declare that they have no competing financial interests. All authors were employed at F. Hoffmann-La Roche Ltd. at the time of work.

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