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Imaging unconditioned fear response with manganese-enhanced MRI (MEMRI)

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Recent use of manganese-enhanced MRI (MEMRI) to assess the neural circuitry involved in autonomic and somatosensory paradigms has been promising. The current study addresses the feasibility of utilizing this technique to assess more complex cognitive and emotional processes. Since olfactory cues are particularly salient to animals, we utilized odorless air, novel/arousing and novel/fear-inducing scents to assess the neural circuitry sub-serving novelty and unconditioned fear. The present imaging data clearly indicate that animals with no prior exposure to a threat-inducing emotional stimulus selectively activated the unconditional fear neuronal pathway, specifically with heightened amygdala and hypothalamic activation. While animals exposed to the novel/arousing compared to fear-inducing odor demonstrated enhanced uptake in the cingulated and prefrontal cortices. In addition, as expected the hippocampus showed significantly enhanced manganese contrast after novelty exposure. Therefore the current study support the validity of MEMRI in the exploration of highly relevant complex neural circuitries associated with cognition and emotion.

Keywords: Manganese; Unconditioned fear; Emotion; MRI; Predator odor; Amygdala; Cognition; Novelty

Introduction

To understand the neuronal, emotional and cognitive components of fear researchers have employed several methods, many directed at evaluating the processes associated with fear conditioning. In such a paradigm a seemingly meaningless stimulus such as tone or light (conditioned stimulus—CS) would be temporally paired with an arousing stimulus (unconditioned stimulus—US). The conditioned stimulus, after several exposures, would retain fear-eliciting properties when presented alone. Several elegant studies utilizing this paradigm have identified brain regions, consistently associated with conditioned fear independent of sensory modality (LeDoux, 2000a,b). Converging evidence indicates that one of the more prominent sites implicated in conditioned fear is the amygdala. Not directly connected to any of the sensory input sites, the amygdala receives sensory information through connections from cortical and sub-cortical sites. The interaction between cortical sites and amygdala facilitates the detection of stimuli from all sensory modalities (LeDoux, 1986, 2000a,b; LeDoux et al., 1990). In addition, sub-cortical routes to the amygdala provide rapid processing and subsequent information update (Li et al., 1996; LeDoux, 1986). Although considerable progress has been made in understanding the neuroanatomy of conditioned fear, unconditioned fear remains relatively under-explored.

Early studies examining the effects of amygdala lesions on the rat response to the presentation of a cat (as an unconditioned fear-eliciting stimulus) showed that these lesions blocked the expression of fear-related responses (Blanchard and Blanchard, 1972). However, others have reported that lesions to the amygdala did not impair the expression of unconditioned fear responses (Antoniadis and McDonald, 2001). There are also controversies about the role of the hippocampus in unconditioned fear responses. While some studies show that hippocampal ablation produced a decrement in defensive immobility (Blanchard et al., 1970), others show that the fear responses were not impaired after hippocampal lesions (Antoniadis and McDonald, 2001). These inconsistencies in the data may be dependent on the size and location of the lesion. At least one study has examined the biochemical effects of unconditioned fear stress compared to conditioned fear (Menon, 2001). Menon reports that the dopaminergic system in the amygdala was activated by unconditioned fear but not by conditioned fear stress.

For most animals, a primary fear-eliciting event is the possibility of being attacked by a predator. Consequently, predator stress (exposure to predator odor) is currently being simulated under laboratory conditions to trigger unconditioned (innate) fear responses in animals (Blanchard et al., 1997). A synthetic compound trimethylthiazoline (TMT) isolated from fox feces has been successfully used as a fear-inducing predator odor (Rosen, 2004), producing increased defensive behaviors and stress hormone release (Morrow et al., 2000; Perrot-Sinal et al., 1999). Interestingly, researchers have also reported that embedded in the fear response is the aspect of the stimulus related to its novelty1 (Williams et al., 2004). Therefore, the theoretical

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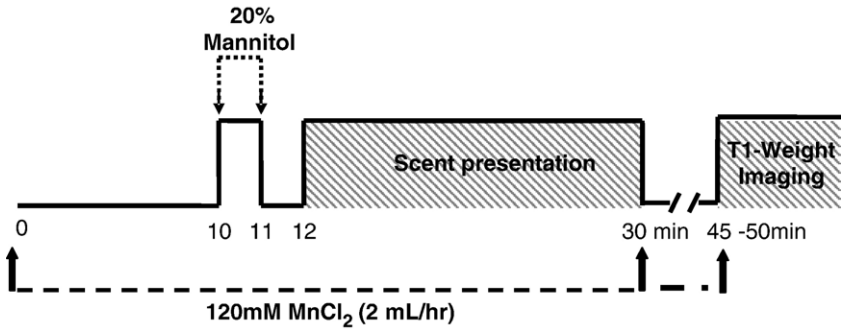

Fig. 1. Experimental design used for manganese administration and scent presentation. Manganese infusion commenced immediately after surgery. After exposure to the olfactory stimulus (lemon scent, fox scent or odorless air), T1-weighted images were acquired on a 4.7 T Bruker Magnet. Image data collection began approximately 15 min post manganese infusion.

framework for the current design incorporates both a novel and fear-inducing odors. The utilization of an olfactory paradigm has provided a unique opportunity to assess the central features of novelty and unconditioned fear response with imaging techniques.

The current study was designed to use manganese-enhanced magnetic resonance imaging (MEMRI) methodology to explore the neural circuitry sub-serving unlearned or innate fear and to visualize emotional responding in an awake animal. MEMRI is one of the more recent MRI modalities being used to map brain activity in a myriad of neuroimaging studies (Duong et al., 2000; Lin and Koretsky, 1997; Silva et al., 2004). MEMRI provides a multiplicity of benefits including the imaging of functional neural circuits via anterograde connections 2–7 (Pautler et al., 1998, 2003; Watanabe et al., 2001), activation-induced accumulation in excitable cells (Aoki et al., 2004; Duong et al., 2000; Lin and Koretsky, 1997) and whole brain contrast (Aoki et al., 2004; Watanabe et al., 2001). Although there are many challenges associated with MEMRI methodology like toxicity (Silva et al., 2004), the distinctive T1 contrast obtained with MEMRI supports excellent visualization of brain cyto-architecture, while the movement of Manganese (Mn^{2+}) across voltage gated calcium channels (Anderson, 1983) facilitates neuronal tract tracing. Furthermore, the unique utilization of MEMRI by Pautler and colleagues (1998, 2003) to tract olfactory processing (up to 48 h after delivery) adds validity to the feasibility of utilizing odors in the current study. Since olfactory cues are critical to decision making in animals particularly rodents, we utilized two unique odors, one a novel/arousing and the other novel fear-inducing along with odorless air (control), to assess the behavioral responses and brain regions pertinent to the processing of cognitive and emotional responses, generated by each scent. We hypothesize that brain activation accompanying each odor will reflect the common as well as distinct brain regions sub-serving the neuronal response to control air as well as arousal due to novelty and unconditioned fear.

Materials and methods

Animals

Adult male Sprague–Dawley rats ($n=5$ for control; $n=5$ for lemon scent; $n=6$ for fox scent) were obtained from Harlan Sprague–Dawley Laboratories (Indianapolis, IN). Animals were housed in Plexiglas cages (two per cage) and maintained in ambient temperature (22–24 °C) on a 12:12 light: dark cycle (lights on at 09:00 hr). Food and water were provided *ad libitum*. All animals were acquired and cared for in accordance with the guidelines published in the NIH *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publications N. 80-23, Revised 1996).

Behavior

Rats were tested to assess their behavioral response to each scent and control prior to imaging studies. Briefly, a separate group of male rats ($n=8$ per group) were removed from their home cage between 9:00–11:00 am EST (to minimize the impact of

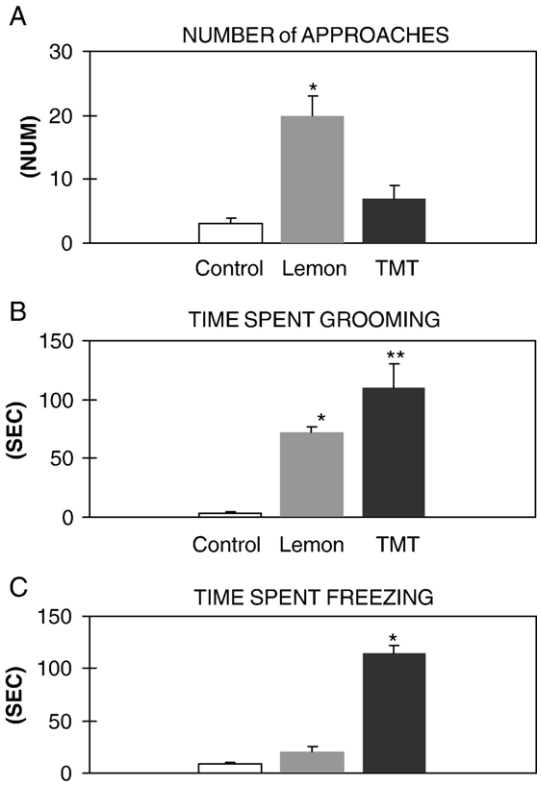

Fig. 2. Behavioral responses to odors. Number of approaches (A), grooming (B) and freezing time (C) were monitored ($n=8$) after exposure to control (no-odor), novel/arousing (lemon) or fear-inducing (TMT) scents. Values are presented as mean ± SEM, statistics different are noted (*$p \leq 0.05$; **$p < 0.01$); NUM (number of movements).

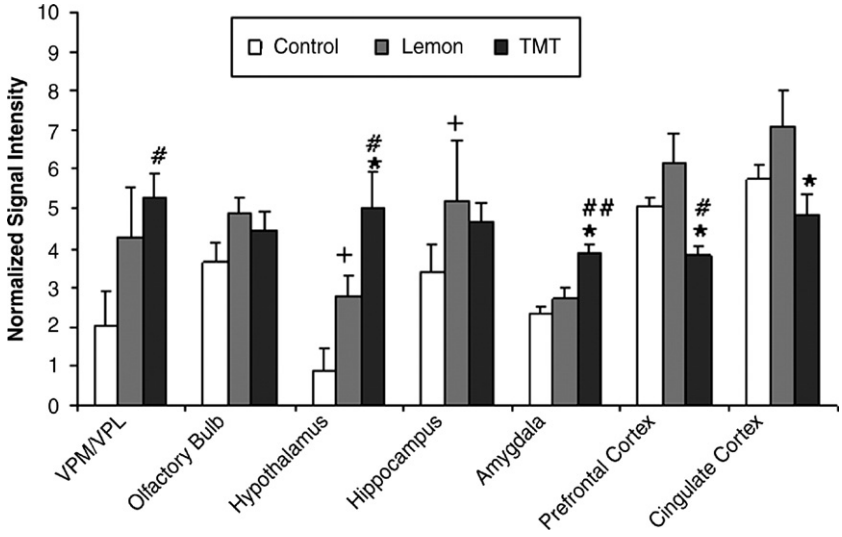

Fig. 3. Signal intensity changes in the ipsilateral hemisphere following control, lemon or fox scent presentations. MRI signal intensity is normalized and averaged for all animals. Values are presented as mean ± SEM. Regions in the lemon scent animals with significant signal and contrast differences compared to control are noted ($^{+}p \leq 0.05$). Regions in the fox scent animals with significant signal and contrast differences compared to control are noted ($^{\#}p \leq 0.05$; $^{\#\#}p \leq 0.01$). Regions in the fox scent animals with significant signal and contrast differences compared to lemon scent are noted ($*p \leq 0.05$; $**p \leq 0.01$). Abbreviation: VPM/VPL—ventral posterolateral/posteromedial thalamus.

circadian rhythms) and allowed to habituate to the environment (plastic container) for two 10 min sessions. The following day, the rats were placed in the same environment and exposed to the different odors. The scents were delivered via an adapted odor meter (ventilator airflow) system. The adapted odormeter system was fitted with porous filter paper containing either 100 μl of water (odorless), lemon or fox scent in the airflow vent at the top (front) of the cage of the rats being tested (5 min). The session was videotaped and scored later for amount of time spent exhibiting exploratory, arousing and defensive behaviors namely approach toward scents, grooming and freezing, respectively.

Acclimation procedure

For imaging experiments, animals were acclimated to the restraint device for 3 days according to a previously published procedure (King et al., 2005). Briefly, animals were lightly anesthetized with 2% isoflurane secured in a dual coil rodent restrainer developed for fMRI (Insight Neuroimaging Systems LLC, Worcester, MA). A plastic semicircular headpiece with blunted ear supports that fit into the ear canals was positioned over the ears. Lidocaine paste (2%) was added to points of mechanical restraint, e.g., bridge of the nose and ear canals to

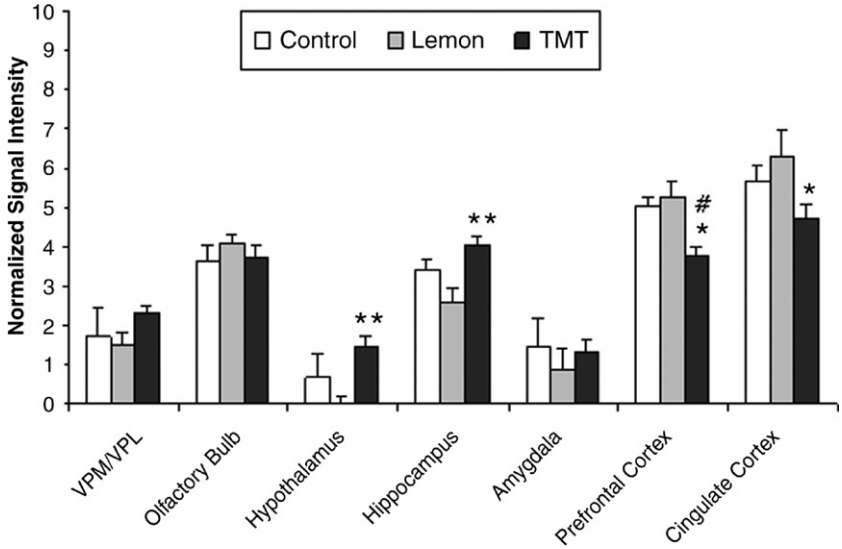

Fig. 4. Signal intensity changes in the contra-lateral hemisphere following control, lemon or fox scent presentations. MRI signal intensity is normalized and averaged for all animals. Values are presented as mean ± SEM. Regions in the lemon scent animals with significant signal and contrast differences compared to control are noted ($^{+}p \leq 0.05$). Regions in the fox scent animals with significant signal and contrast differences compared to control are noted ($^{\#}p \leq 0.05$; $^{\#\#}p \leq 0.01$). Regions in the fox scent animals with significant signal and contrast differences compared to lemon scent are noted ($*p \leq 0.05$; $**p \leq 0.01$). Abbreviation: VPM/VPL—ventral posteromedial/posterolateral thalamus.

minimize any pain or discomfort during the study. The head was placed into the cylindrical head holder with the animals' incisors secured over a bite bar and the ears were positioned inside the head holder. The body of the animal was placed in a custom-fitted cylindrical body tube. The body restrainer isolates all of the body movement from the head restrainer and minimizes motion artifact (Lahti et al., 1998, 1999), while allowing for unrestricted respiration. The head holder and body tube were subsequently placed in a black opaque tube "mock scanner" with a tape-recording of scanner noises. Scanner noises were identical to the precise imaging protocol to which rats would later be exposed during the experimental imaging protocol.

Cannulation and injection of $MnCl_2$

On the day of the experiment, rats were anesthetized with 2%-isoflurane for surgery. Polyethylene catheters (PE-50) were placed in the femoral vein and the right common carotid artery (CCA). Mannitol could later be administered from the severed CCA toward the internal and carotid artery (ICA) and brain but not toward the heart. After surgery, isoflurane was discontinued and animals were returned to their adapted home-cages awake (with odor meter) for scent and Mannitol administration.

Rats were infused in the femoral vein with 120 mM $MnCl_2$ at a rate of 2 ml/h for a total of 30 min in their home cage. Ten minutes after starting the infusion, a bolus of 20% D-mannitol (at 4 °C, dissolved in 0.1 M PBS, pH 7.4) was given into the right carotid artery at a concentration of 5 ml/kg via the prepared catheters. Mannitol was injected at a constant rate over 1 min to disrupt the blood brain barrier (BBB). One minute after the mannitol injection, rats were exposed to either odorless air (control), lemon (novel/arousing) or TMT (fear-inducing stimulus) via the odor meter attached to the entrance of the home cages until the end of the 30 min infusion period (Fig. 1).

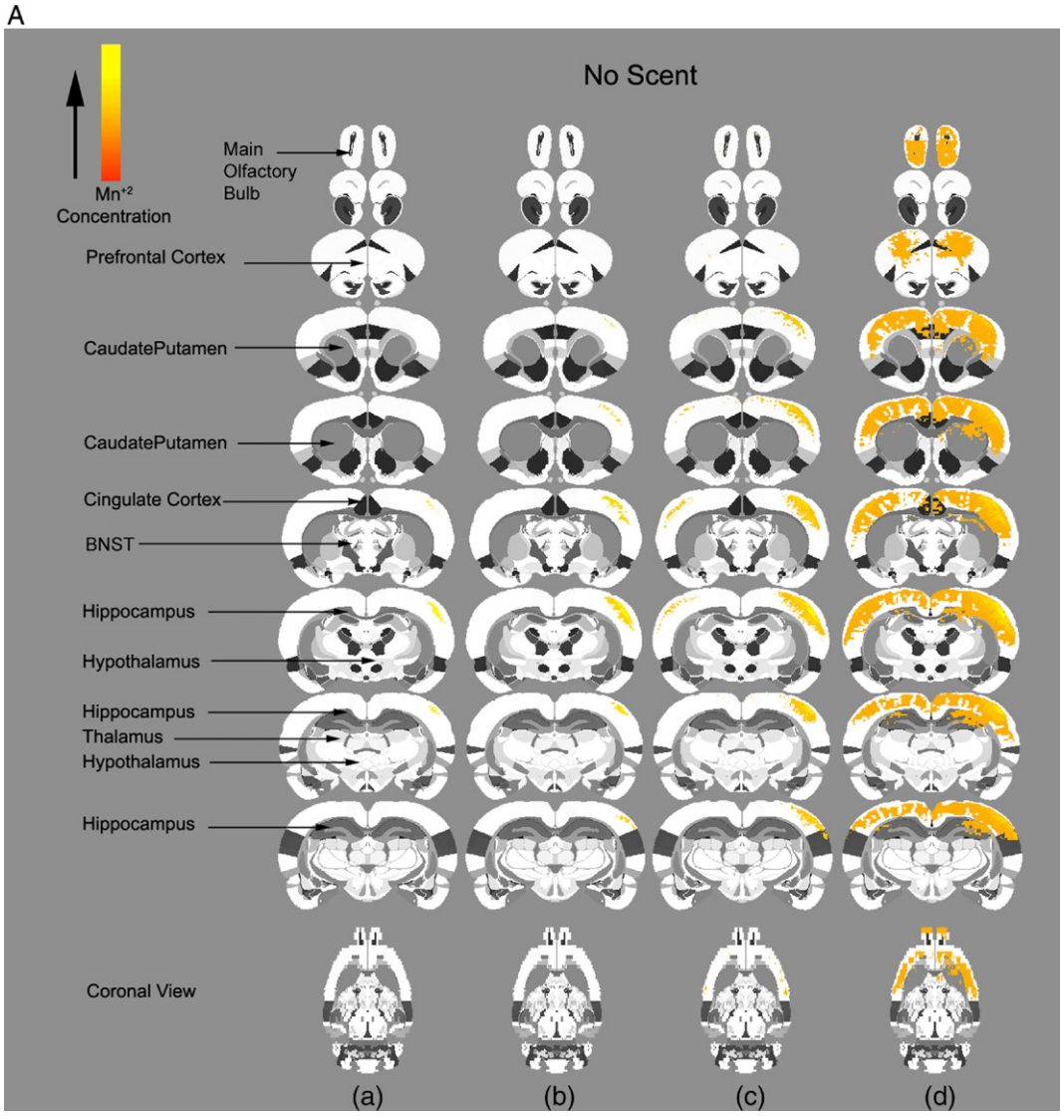

Fig. 5. Manganese-enhanced T1-weighted MR images. T1-weighted MR images of representative rats after exposure to odorless air/control (A), lemon (B) and fox scent (C). Images were acquired 45 min after unilateral (right hemisphere) infusion of $MnCl_2$. These coronal sections reveal differential signal enhancement in cortical and sub-cortical sites on both the ipsilateral and contralateral hemispheres sub-serving emotional responses to control air, novelty and unconditioned fear.

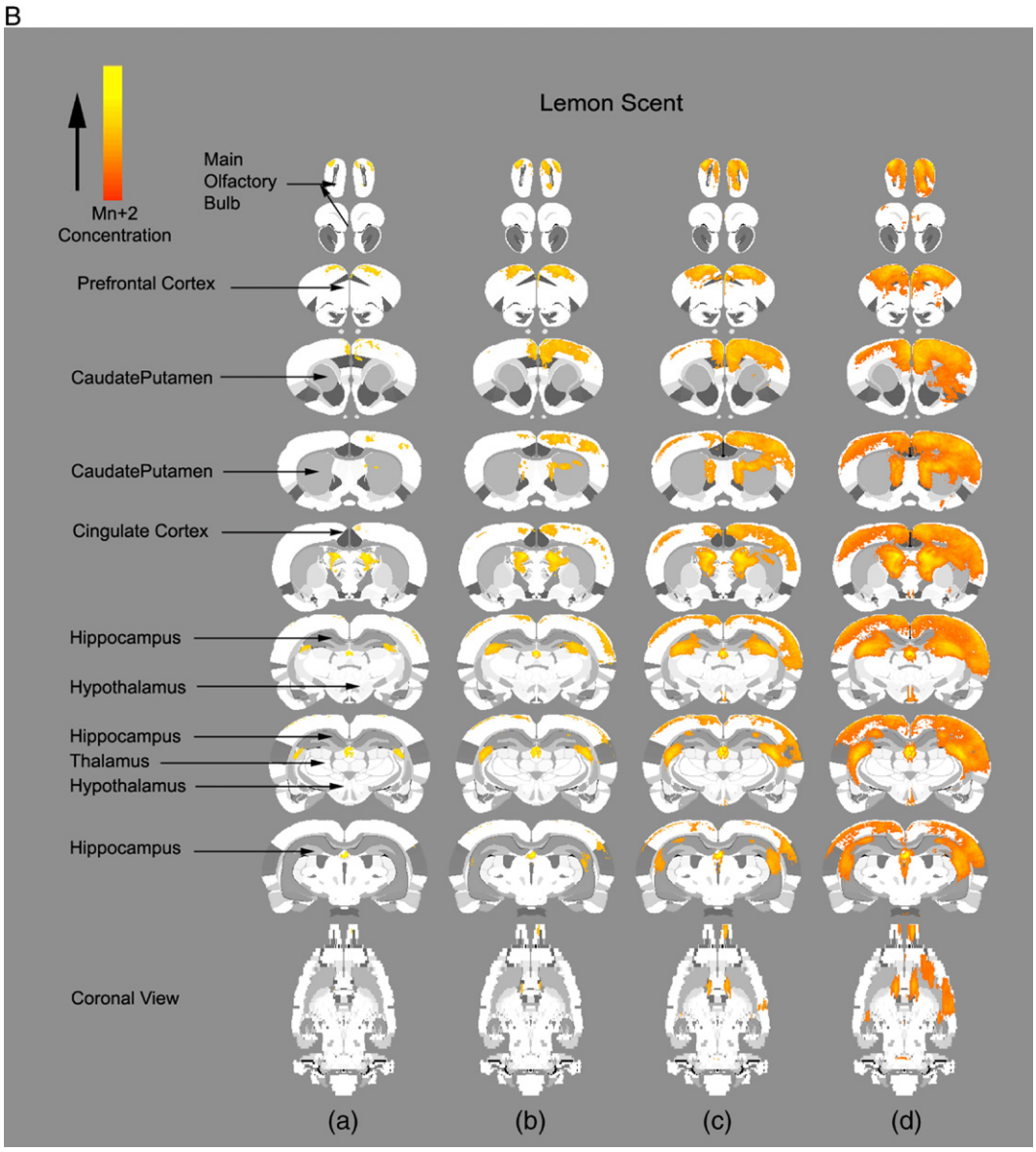

Fig. 5 (*continued*).

Magnetic resonance imaging

After the infusion of $MnCl_2$ rats were secured in a MR compatible restrainer as stated above (see Acclimation procedure). All images were acquired using a 4.7T/40 cm horizontal magnet equipped with a Bruker BioSpec console and a 20 Gauss/cm magnetic field gradient insert (inner diameter, 12 cm) capable of a 120 μs rise time (Bruker, Billerica, MA U.S.A). High resolution multislice anatomical data sets were acquired using a fast spin echo pulse sequence (RARE, or rapid acquisition relaxation-enhanced) with TR = 2.0 s, effective TE = 12 ms, Matrix = 256 × 256, FOV = 2.5 × 2.5 cm, number of averages = 12, eighteen 1.0-mm thickness slices, at the beginning and end of each imaging session. Subtraction of these data sets confirmed there was no significant movement of the animal over the imaging session. T1-weighted images were acquired 45–50 min after the commencement of $MnCl_2$ infusion (Fig. 1). Images were collected with a gradient echo sequence: TR = 300 ms, TE = 4.2 ms, Matrix = 256 × 256, number of averages = 12 and with the same geometry parameters as the anatomical images. The shortening of T_1 results in MRI images exhibiting positive contrast enhancement in tissues with Mn^{2+}accumulation.

Data processing

Signal intensities in specific regions of interest (ROIs) were measured using STIMULATE software (Strupp, 1996). In order to control for individual variation between rats, the values for each ROI were normalized within subject by utilizing brain regions devoid of manganese uptake (in all animals studied independent on scent utilized the pons and pontine nuclei) as background or baseline signal intensity. Data from composite ($n = 5$/no odor; $n = 5$/lemon scent; $n = 6$/TMT) were compared and analyzed using the unpaired Student's *t*-test.

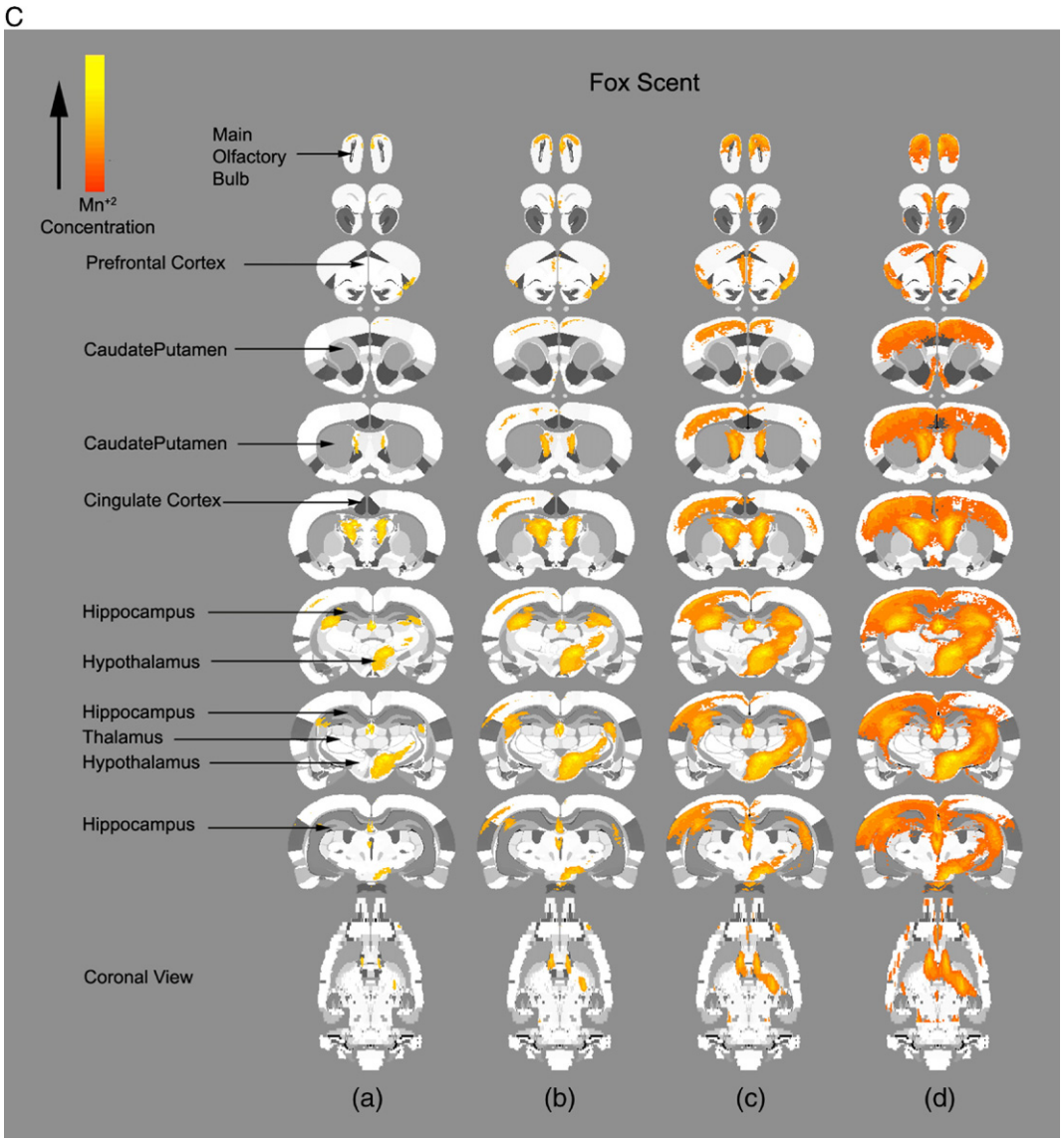

Fig. 5 (*continued*).

Areas with enhanced contrast were visualized in atlas format using MIVA (Medical Image Visualization and Analysis. http://ccni.wpi.edu/) To compare no odor, novel/arousing and novel/fear group data sets, MRI image data were registered to the rat brain atlas and the various threshold values were examined to selectively visualize Mn^{2+} accumulated regions (high intensity areas) on the foreground (gray scale) of atlas (Figs. 5A–C).

Results

Behavioral responses

Fig. 2 summarizes behavioral (cognitive and emotional) responses to odors (control (odorless), novel/arousing (lemon) and novel/fear-inducing (TMT)) as monitored by changes in number of approaches, grooming episodes and freezing behavior (Fig. 2A, number of approaches). As expected, animals spent significantly less time in exploration and investigation of the novel/arousing scent ($p \leq 0.01$) than the novel/fear inducing scent or no odor/control. In addition, novelty-induced grooming response, an indicator arousal (Komorowska and Pellis, 2004), was increased in response to lemon and TMT as compared to no odor ($p \leq 0.05$; $p \leq 0.01$; respectively). Finally, animals spent a significant amount of time freezing to TMT compare to either the no-odor/control or lemon scent ($p \leq 0.05$). Freezing is a critical defensive behavioral manifestation of fear response in rodents (Blanchard et al., 1970).

After scent exposure, it was noted that lemon odor was associated novelty-induced and arousal makers, while the TMT odor correlated with changes in both arousing and defensive (fear) behaviors. These scents and no/odor controls were utilized in following MEMRI studies.

Uptake of Mn^{2+} by ipsilateral hemisphere

Fig. 3 represents the normalized and averaged signal intensities (mean±SEM) for each region of interest (ROI) in the ipsilateral

hemisphere following odor-meter exposure. All animals exposed to the odor-meter and presented with either odorless air (control), lemon scent (novel/arousing stimulus) or TMT scent (novel/fear-inducing stimulus) exhibited positive regional contrast enhancement in the olfactory bulb.

The presence of manganese was observed following exposure to airflow independent of the scent, in regions of the olfactory bulb, thalamus, hippocampus, amygdala and cortex. Significant differences in manganese signal intensity observed with the novel/arousing (lemon) olfactory stimulation versus no odor controls were observed the hippocampus and hypothalamus ($p<0.05$). While differences in enhanced signal intensity observed in TMT (novel/fear-inducing) versus odorless control scent were observed in the thalamus ($p<0.05$), hypothalamus ($p<0.05$) and amygdala ($p<0.01$). This was also accompanied by diminished manganese signal intensity in the prefrontal cortical region of the TMT versus odorless control ($p\leq0.05$) experimental group. Finally, there were distinct differences in manganese signal intensity with novel/fear inducing versus novel/arousing odors. While novel/arousing (lemon) olfactory stimulation resulted in significant increases ($p\leq0.05$) in average signal intensity, the prefrontal and cingulate cortical regions compared to the fear-inducing predator odor, TMT exposure was accompanied by dramatic increases in signal intensity in the amygdala and hypothalamus ($p\leq0.05$) when compared to manganese uptake in lemon and control groups.

Uptake of Mn^{2+} by the contra-lateral hemisphere

Although the BBB was disrupted in the ipsilateral hemisphere movement of Mn^{2+} across the commissural fibers and ventricles to the adjoining hemisphere was apparent. Fig. 4 depicts the normalized and averaged signal intensities (mean±SEM) for each region of interest in the contra-lateral hemisphere following no-odor, lemon and fox scent exposure.

All animals exposed to odor meter exhibited positive regional contrast enhancement or the presence of manganese in the olfactory bulb in the contra-lateral hemisphere (hemisphere opposite to infusion of contrast). The presence of manganese contrast as compared to background was also clearly visualized within the cortex, thalamus, hypothalamus, hippocampus, and amygdala following presentation of all animals. There were no differences between the novel/arousing scent and odorless air (control). However there were distinct differences in manganese signal intensity with novel/arousing versus fear-inducing odors. Novel/arousing olfactory stimulation resulted in significant increases ($p\leq0.05$) in average signal intensity the prefrontal and cingulate cortical regions compared to the fear-inducing (TMT) predator odor, while TMT was accompanied by dramatic increases in signal intensity in the hypothalamus and hippocampus ($p\leq0.01$) compared to the lemon odor exposure.

Visualization of manganese contrast

Two- and three-dimensional image data sets allowed for the $MnCl_2$-dependent enhancement to be viewed in axial, sagittal and horizontal planes. Representative 2D T1-weighted images of nine consecutive brain slices in a representative animal from one exposure of either lemon or fox odor are depicted in Fig. 5. As noted in Figs. 3 and 4, the presence of manganese uptake was observed, 45 to 50 min after the commencement of manganese infusion, in several brain regions ipsilateral and contralateral to the site of injection as hyperintensity in T1-weighted images. Mn^{2+} uptake is represented in a contrast-dependent manner with threshold data from the most stringent threshold intensity ($p\leq0.01$) to the least ($p\leq0.05$) in a step-wise manner (Figs. 5A, (a)–(d)). Given the assumption that the rate of manganese uptake rate is consistent in a neuronal population then brain regions with the highest threshold represent more selective manganese accumulation over time. Consistent with the composite graphic data set (Figs. 3 and 4), the 2D multislice representational atlas images (depicted in black grey and white) reveal activation (brightness in image-depicted as changes in color bar) in a representative animal in selected brain regions after exposure to odorless air, lemon or TMT odor, respectively (Figs. 5A, (b) and (c)).

In control animals there was nominal manganese uptake visualized at the most stringent (Figs. 5A, (a)–(c)) while enhanced manganese contrast is visible in the olfactory bulb and cortical regions after exposure to odorless air at the least stringent intensity threshold (Fig. 5A, (d)). As expected from the composite data set enhanced manganese contrast is visible in the olfactory bulb after lemon odor exposure at the most stringent intensity threshold (Fig. 5B, (a)). The hippocampus and dispersed cortical regions were visible at the next significant intensity threshold level (Figs. 5B, (b) and (c)). While, at the least stringent but significant threshold value (Fig. 5B, (d)), the lemon exposed rats had significant levels of manganese in the ventricles, amygdala, thalamus and hypothalamus. Similarly in the fox scent exposed animals at the most stringent intensity threshold manganese contrast is visible in the olfactory bulb (Fig. 5C, (a)). However, cortical activation is limited in the region of the cingulate cortex (Fig. 5C, (b)). The ventricles and hippocampus were visible at the next significant intensity threshold level (Fig. 5C, (c)). Finally, the distinct deeper limbic brain regions that accumulated manganese, in response to the fear-inducing odor, were the hypothalamus and amygdala (Fig. 5C, (d)).

Taken together, the manganese uptake from olfactory cues utilizes a circuitry that engages the olfactory bulbs followed by cortical sites, ventricles and finally sub-cortical sites.

Discussion

These current data support other recent studies in which manganese-enhanced magnetic resonance imaging has been successfully utilized to trace odor-induced brain activation (Pautler et al., 2003) and visualize brain anatomy in vivo (Aoki et al., 2004; Watanabe et al., 2004). In addition, the utilization of odorless air as a control demonstrated that brain activation occurs in several regions reflective of a novel occurrence. Response to novelty has been extensively utilized in cognitive neuroscience to assess the contribution of different brain regions to aspects of attention, and investigation of salient stimuli (Yamaguchi et al., 2004; Yamasaki et al., 2002; Jessen et al., 2002). The rapid assessment of novelty generally relies on the processing of new information against stored sensory information. As such neurons in the hippocampus intricately involved in certain forms of learning have been implicated in novelty detection (Rutishauser et al., 2006; Nyberg, 2005; Kohler et al., 2005; Meltzer and Constable, 2005). The most notable differences in brain activation accompanying novelty were found in the hippocampus and cortex, with the prefrontal and cingulate cortex preferentially activated after novelty exposure as opposed to TMT. This is consistent with the premise that novelty detection involves not just the hippocampus but higher cortical regions (Kohler et al., 2005; Downar et al., 2002) recruited for

selective attention, and processing of unexpected novelty (Yamaguchi et al., 2004). In addition, the behavioral studies suggest that lemon scent may be novel, as well as arousing, again this would be in accordance with the work of others (Kuo and Yen, 2005) demonstrating the involvement of the cingulate cortex in arousing/novel stimulus processing. Furthermore, the selective manganese contrast observed in the hypothalamus, a region intricately linked to arousal (de Lecea et al., 2006; Suntsova et al., 2007) after lemon exposure supports the hypothesis that lemon scent is both novel and arousing.

In accordance with the distinctly different behaviors elicited by the odors, manganese accumulation after fear-eliciting odor (Rosen, 2004; Blanchard et al., 1997), resulted in enhanced defensive fear behavior and accompanying brain activation in cortical and sub-cortical sites predicted by methods like high density FOS immunoreactivity (Dielenberg and McGregor, 2001). Our data suggest that unconditioned fear from the ethnologically relevant threat of a predator may share several commonalities including the behavioral response and underlying neural circuitry (independent of the identity of the predator utilized cat versus fox). In addition, these studies support a degree of redundancy in the circuitry sub-serving sensory cues derived from both the novel/arousing and novel/fearful scents. This is not unexpected since others have shown that the fear response is a composite of stimulus novelty and arousing emotional content of the stimulus (Williams et al., 2004).

In fact the enhanced manganese accumulation in cortical and amygdala regions after TMT exposure may lend support to the hypothesis that the fear response is characterized as a persistent stressor (Williams et al., 2004), with the magnitude of the brain activation being sustained by the reciprocal feedback system between the cortex and amygdala (Hariri et al., 2003). From anatomical studies, it is apparent that there is a rapid neuronal circuitry to process fearful olfactory cues. Olfactory projections to cortex involve both monosynaptic projections (which is subsequently reciprocally connected to the amygdala) and direct dense monosynaptic input with the amygdala complex. Both behavioral and neuropsychological reports suggest that the cortex is involved in establishing associations between odors and emotional stimuli and acts as an important relay station for such information (Otto et al., 2000).

Taken together, these results clearly indicate that the novel/arousing odor resulted in activation of the classical neuronal circuitry attributed to odor processing, arousal and novelty, while the threatening/fearful olfactory scent elicited more activation in the amygdala and hypothalamus. Since, the amygdala may be both directly and indirectly involved in the neurological components of the fear response and the hypothalamic–pituitary–adrenal (HPA) axis, this may be another feedback loop. The observed neuronal specificity accompanying exposure to an unknown fear-inducing odor suggests an innate cognitive and emotional response that is selective to threat not simply arousal. Finally, the utilization of manganese-enhanced MRI for circuitries sub-serving emotional and cognitive processing is now apparent.

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