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Manganese-Enhanced MRI Reveals Brain Circuits Associated with Olfactory Fear Conditioning by Nasal Delivery of Manganese

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Purpose: The survival of organisms critically depends on avoidance responses to life-threatening stimuli. Information about dangerous situations needs to be remembered to produce defensive behavior. To investigate underlying brain regions to process information of danger, manganese-enhanced MRI (MEMRI) was used in olfactory fear-conditioned rats.

Materials and Methods: Fear conditioning was conducted in male Sprague-Dawley rats. The animals received nasal injections of manganese chloride solution to monitor brain activation for olfactory information processing. Twenty-four hours after manganese injection, rats were exposed to electric foot shocks with odor cue for one hour. Control rats were exposed to the same odor cue without foot shocks. Forty-eight hours after the conditioning, rats were anesthetized and their brains were scanned with 9.4T MRI. Acquired images were processed and statistical analyses were performed using AFNI.

Results: Manganese injection enhanced brain areas involved in olfactory information pathways in T1 weighted images. Rats that received foot shocks showed higher brain activation in the central nucleus of the amygdala, septum, primary motor cortex, and preoptic area. In contrast, control rats displayed greater signals in the orbital cortex and nucleus accumbens.

Conclusion: Nasal delivery of manganese solution enhanced olfactory signal pathways in rats. Odor cue paired with foot shocks activated amygdala, the central brain region in fear, and related brain circuits. Use of MEMRI in fear conditioning provides a reliable monitoring technique of brain activation for fear learning.

Keywords: Manganese MRI; Fear conditioning; Olfactory circuit

INTRODUCTION

The core functions of life are survival and reproduction. Avoidance of danger is a critical ability to survive. Most animals inherently evade their natural enemies. It

is also very useful for survival that they can learn and remember the moment when they confront danger. The faster memories of dangerous situations are formed and remembered for a long time, the more beneficial it will be to avoid future risks. For memory formation by a momentary experience, the sensory information experienced in a dangerous situation is vividly activated. Then, it produces the negative emotion of fear. Fear response enables animals avoid frightening situations, so fear plays a key role in survival (1).

The fear response is not only very widely studied in emotion research but also extensively used to study learning and memory mechanisms in laboratory animals (2). For example, the popular Pavlovian fear conditioning procedure is as follows. Rats (or mice) are placed in a small box and exposed to a harmless sensory stimulus (e.g., sound, smell, light, etc.) for 10-30 seconds. At the end of the stimulus, a momentary electric foot shock (0.5-1 mA) is administered to metal grids at the bottom of the box. Several pairings of sensory stimulus and electric shock produce conditioned fear response, which is freezing behavior to initially neutral stimulus and the trained context (e.g., an experiment box). The memory of conditioned fear lasts a week to several months and does not disappear easily, which causes post-traumatic stress disorder in severe cases.

The critical brain area responsible for fear memory is known as the amygdala (3). The amygdala is essential for learning fear responses. When this area is damaged or inhibited, fear learning does not occur, and memory is not formed. The amygdala also plays a key role in memory storage, so damaging or inhibiting the amygdala does not produce a fear response. In a typical fear conditioning setup, auditory tones are paired with a foot shock because the experimenter can easily control sound with a personal computer. Rats and mice, on the other hand, are nocturnal animals with extremely sensitive hearing and smelling senses. In nature, the odor is a critical signal in recognizing natural predators. Also, odor molecules are transmitted directly to the brain, and the olfactory pathway is very closely connected to the amygdala. However, olfactory fear conditioning has not been extensively investigated, especially in MRI studies.

MRI-based brain imaging techniques are widely used to monitor the activation of the entire brain in humans and animals. In particular, functional MRI techniques, which measure blood oxygenation level-dependent (BOLD) signals to observe activation areas of the brain, are commonly applied for monitoring brain functions. BOLD MRI is also widely used for animal study, however, most brain imaging in animals is performed under anesthesia. High-resolution brain imaging of small animals requires high-field MRIs (e.g., 7T or higher tesla). Since a high field magnet has a small bore (usually ~ 20 cm diameter), it is almost impossible to simultaneously observe brain activation while performing behavioral tests in the magnet. Another method for measuring brain activations is the MEMRI technique. The paramagnetic manganese ion, as an effective contrast agent, accumulates in neurons via voltage-gated calcium channels and enhances contrast in the activated tissues captured by T1 weighted MRI (4-7). In the MEMRI experiment, manganese solution is first administered to an animal. When manganese diffuses into the brain, the animal is exposed to experimental conditions or behavior treatment. One or two days after manganese injection, the animal brain is scanned to monitor brain activation. T1 signal difference reflects brain activities depending on the animal's prior experience. Depending on manganese injection routes, MEMRI can be used to trace brain networks. For example, manganese is administered to the eye and used for tract-tracing of neurons from the eyeball to the brain (8). Nasal infusion of manganese is used to monitor olfactory signal pathways to the brain (9).

In this study, we examined brain activation related to fear conditioning by MEMRI. The nasal manganese infusion method was used to investigate olfactory signal pathways involved in fear memory formation. Manganese not only accumulates in the primary administration site but also is transmitted through connected synapses in various parts of the brain. Since the accumulation of manganese enhances the contrast of the MRI T1 image, we can compare the degree of brain activation related to fear memory.

MATERIALS AND METHODS

Animals

Animal handling and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Daegu-Gyeongbuk Medical Innovation Foundation (DGMIF). Male Sprague-Dawley (SD) rats (six-seven weeks old; weighing 160-240 g) were housed three animals per cage. The vivarium has a constant temperature (20-25°C) and humidity (40-45%) controlled and 12:12 hours light/dark cycle maintained. Rats freely accessed normal rodent pellets and water in their home cages during the entire experiment.

Manganese Administration

Rats were anesthetized with 4% isoflurane and maintained with 2% isoflurane. Manganese chloride dissolved in saline ($MnCl_2$, 0.2 mmol/kg body weight) was injected with a polyethylene catheter (PE10) tubing connected to a Hamilton syringe. The tip of the tubing was 1.5 mm pushed into the nasal cavity. After rats were recovered from anesthesia, they were individually placed into a clean cage with free access to water only.

Fear Conditioning Procedure

Fear conditioning was performed in a cube box consisting of two transparent plastic walls and two aluminum walls (30.5 × 25.4 × 30.5 cm; Coulbourn Instruments), in a separate sound-attenuating chamber. The floor of a conditioning box has consisted of a series of steel rods through which electric current flows. Each rod is spaced about 5 mm apart. A video camera was installed on the ceiling to record rat behavior.

One day after nasal manganese infusion, rats were placed in the conditioning box for one hour. Odor cue, 100 µl linalool solution (sigma-ALDRICH, 97%, China), was sprayed over the paper towel below steel rods. Fear conditioning rats (n = 6) were received a total of 20 electrical foot shocks (0.5 sec long and 1 mA) delivered once every three minutes. Control rats (n = 6) stayed in the same box without foot shocks. Afterward, rats were returned to their home cages (Fig. 1).

MRI Scanning

MRI scanning was conducted 48 hours after administration of manganese infusion. The rat was anesthetized with isoflurane (4% induction, 1.5% maintenance) and placed in a plastic bed equipped with a temperature-controlled warming blanket. The rat head was covered with Bruker 4-channel phased array surface coil for brain imaging. An 82 mm inner diameter quadrature volume coil was used for RF transmission. MRI scanning was performed on a 9.4T MRI scanner (Bruker, BioSpec 94/20 USR, Germany). 2D spin-echo was used for acquisition of T1-weighted images, sequence parameters were as follow: field of view, 35 × 35 mm matrix size, 256 × 256 axial slices, 1 mm slice thickness, no gap, repetition time (TR) = 1500 ms, echo time (TE) = 6.5 ms, number of acquisitions (NEX) = 6, scan time = 7 min 12 sec.

Image Processing and Statistical Analysis

All image processing and statistical analysis were performed using AFNI (10) and FSL (11). AFNI "3dUnifize" function was performed to remove MR signal intensity gradients difference of the brain image acquired by Bruker 4 channel surface array coil. Individual rat brain image was co-registered to reference high-resolution rat brain template (SIGMA InVivo Brain Template, NITRC: NeuroImaging Tools & Resources Collaboratory, https://www.nitrc.org/projects/sigma_template) using FSL "flirt" function. The coregistered images were then spatially smoothed by 0.45 mm FWHM Gaussian kernel. Between-group comparison was performed using AFNI "3dttest++". The average intensity difference

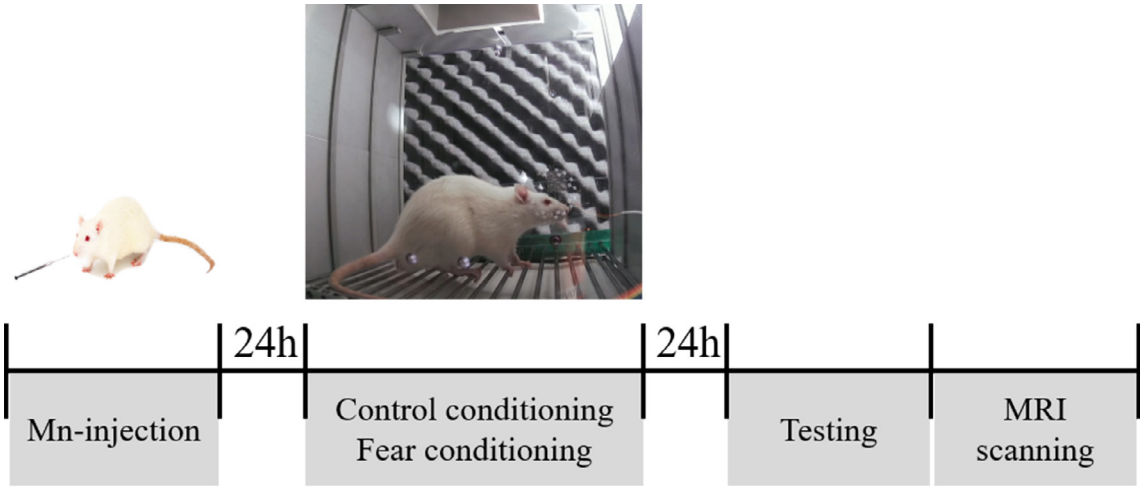
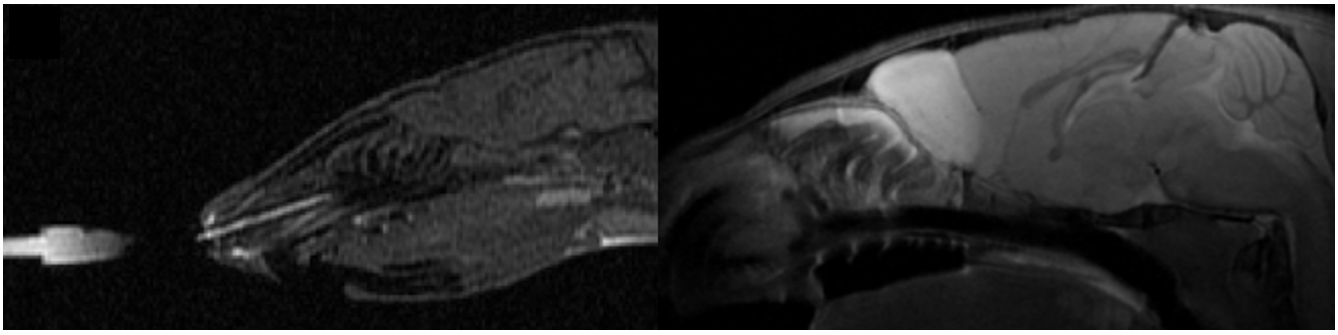
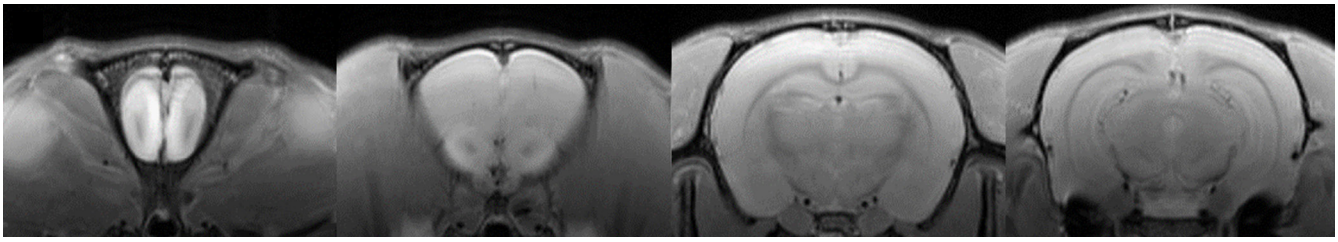


Fig. 1. Experiment design. Manganese solution was administered into the bilateral nasal cavity. One day after manganese injection, fear (or control) conditioning was conducted. On the following day, a test session and MRI scanning were performed.



a



b

Fig. 2. (a) T1 weighted sagittal images were taken before and after administration of $MnCl_2$ into the nasal cavity. (b) Coronal brain slices of T1 weighted MRI. From the left to right four images show brain slices containing the olfactory bulb, orbital cortex, amygdala, and hippocampus.

Table 1. Statistical Analysis Result of the Difference between the Control Group and Fear Group

Regions of interest	P value	Mean of control	Mean of fear	Difference	t value	q value
Orbital cortex	0.000218	942.0	909.8	32.17	5.631	0.000073
Nucleus accumbens	0.000074	921.8	906.0	15.83	6.449	0.000038
Septum	0.000713	874.2	899.5	-25.33	4.810	0.000144
Primary motor cortex	0.000903	978.7	999.2	-20.50	4.654	0.000152
Caudate putamen	0.217321	883.0	888.8	-5.833	1.317	0.031356
Preoptic area	0.000074	881.5	917.5	-36.00	6.442	0.000038
Central nucleus of amygdala	0.000440	916.8	931.2	-14.33	5.136	0.000111

map between fear and control group was created based on "3dttest++" output thresholded by P-value 0.005. The ROI was drawn based on the Paxinos rat brain atlas (12), and the average value of each region was extracted using AFNI "3dROIstats". Between-group comparison (t-test) was performed using Graphpad Prism 9 (GraphPad Software, CA, USA).

RESULTS

Figure 2a shows sagittal images of the rat brain during manganese injection and 48 hours after injection. The olfactory bulb is significantly enhanced. Figure 2b images are four coronal sections of the rat brain showing the olfactory bulb, orbital cortex, amygdala, and hippocampus.

The whole-brain activation map was computed by AFNI "3dttest++". Figure 3 shows the group brain intensity difference map generated by subtracting the average intensity of control rats from that of fear-conditioned rats. Red and yellow color means greater signals in fear-conditioned rats compared to control rats. On the contrary, the blue color indicates higher signals in control rats than those in fear-conditioned rats. Signals from the orbital

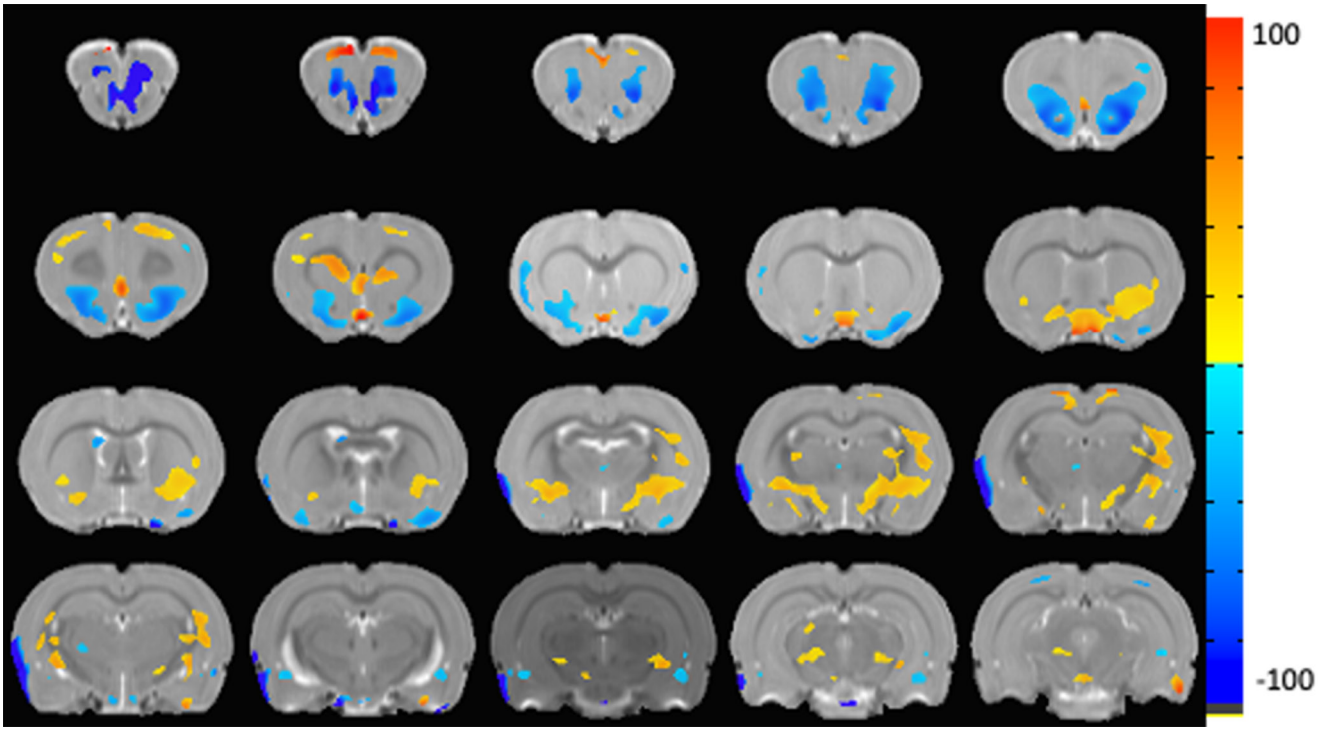


Fig. 3. Whole-brain average intensity difference map (fear - control) threshold by t-test P-value (P = 0.005). The color bar represents signal intensity difference from -100 to 100 (A.U.). Warm colors indicate greater signals in the fear group and cold colors show greater signals in the control group.

cortex, ventral caudate-putamen, and nucleus accumbens were higher in the control rats. On the other hand, signals from the septum, primary motor cortex, preoptic area, central nucleus of the amygdala showed greater in fear-conditioned rats.

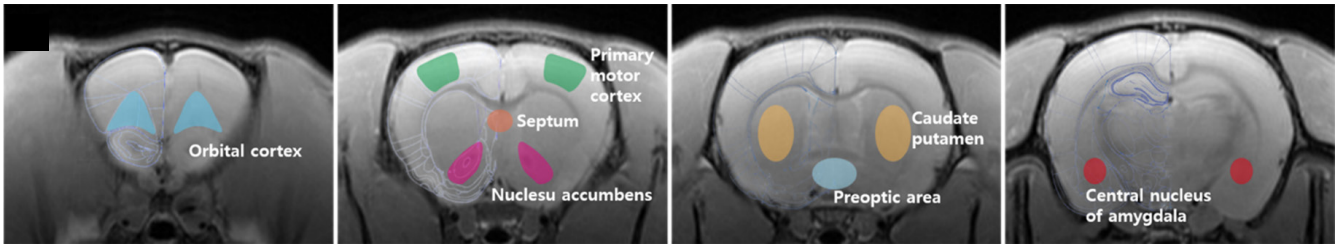
Figure 4a shows locations of seven regions of interest (ROI) such as the orbital cortex, nucleus accumbens, septum, primary motor cortex, caudate-putamen, pre-optic area, and central nucleus of the amygdala. The average signal intensity of the orbital cortex and nucleus accumbens was significantly higher in the control rats than in the fear-conditioned rats ($P < 0.001$). In contrast, signals from the primary motor cortex, preoptic area, and central nucleus of the amygdala were greater in the fear-conditioned rats ($P < 0.001$). There was no significant difference in the caudate-putamen between the groups ($P > 0.05$). The statistical results were summarized in the Figure 4b and Table 1.

DISCUSSION

Analysis of T1 MRI images showed that manganese infused into the mouse nose highly accumulated in the olfactory bulb and adjacent brain regions, which result confirms an earlier MEMRI study (13). Fear conditioned rats displayed greater signal intensity in the amygdala, septum, primary motor cortex, and preoptic area. In contrast, control animals showed greater signals in the orbital cortex and nucleus accumbens.

Odor, a small odorant molecule present in the air, stimulates the olfactory receptor and transmits signals directly to the brain through the olfactory bulb. It connects to the olfactory cortex that is connected to the orbital cortex via the insular cortex (14). The mitral cell of the olfactory bulb connects to the amygdala via the anterior olfactory nucleus and the piriform cortex (15). The tufted cell of the olfactory bulb projects to the anterior olfactory nucleus and olfactory tubercle that is also connected to other brain regions such as the nucleus accumbens, caudate putamen, and lateral septum (16). Matyas and colleagues (17) reported that the amygdala is connected to the motor cortex, orbital cortex.

The accumulation of manganese in the amygdala, a key structure in fear conditioning, increased in foot shocked rats compared to animals not shocked, indicating that fear conditioning was formed. In this experiment, rats



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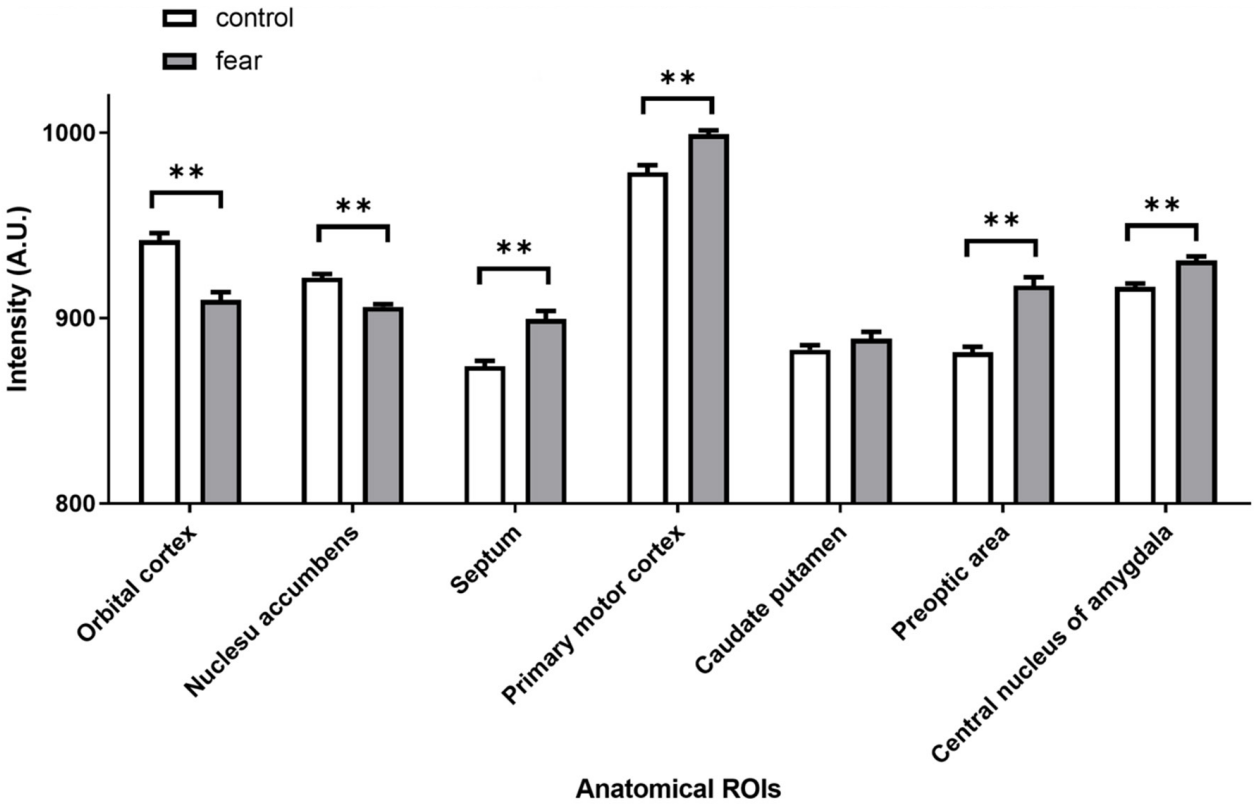

b

Fig. 4. (a) Color regions show anatomical ROIs to compute average manganese intensity. ROIs include the orbital cortex, nucleus accumbens, septum, primary motor cortex, caudate-putamen, pre-optic area, central nucleus of the amygdala. The ROIs were drawn based on the Paxinos rat brain atlas. (b) Average signal intensity of each ROI. The white bar represents the signals from the control group, and the grey bar represents the signal strength of the fear group. **P < 0.001.

received 20 foot shocks. This procedure produces not only fear conditioning but also acute stress. Acute and strong stress causes an aversive response (i.e., fear response) so the results of this study are also linked to the results of the stress experiment. Since olfactory stimulation reminds the context in which acute stress is experienced, the results can be regarded as monitoring brain activity that evokes fear or stress experiences. Several brain regions involved in stress showed different activations between the shocked group and the control group of this study. The orbital cortex (or orbitofrontal cortex) is known to be responsible for negative emotions in humans and to be associated with stress in mice. In addition, the orbital cortex and amygdala network circuits are important in stress responses (18). Inactivation of the orbital cortex - amygdala circuit reduced the stress response. In this experiment, the activity of the orbital cortex was greater in the control, but the activity of the amygdala was increased more in foot-shocked rats. The amygdala, which has a connection to the nucleus accumbens, modulates stress-related behaviors through

reward circuits (19). As Chang and Grace (20) found chronic mild stress decreased dopamine neuron activities, we found reduced manganese accumulation in the nucleus accumbens in foot shocked rats. Bangasser and colleagues (21) reported that repeated social stress enhanced manganese signals in the septum and motor cortex. Anxiogenic stressors prolonged responses in mouse preoptic neurons which correspond to greater manganese signals in the preoptic area in the rats (22).

Manganese injection into the nose visualized the activation of a neural circuit connected to the olfactory bulb via an adjacent synapse. The concentration of the absorbed manganese decreased every time it passed through synapses in the neurons to which manganese was initially administered, so the manganese uptake was lower in sites that passed through several synapses. Therefore, it is difficult to observe activation by fear conditioning in such areas. On the other hand, from the viewpoint of only monitoring neural activation by odor information absorbed through the nose, direct administering manganese only to the nose may be an advantage. Other areas that are not directly related to odor are not measurable because the absorption of manganese is lower. This technique is suitable for tracing olfactory information flow from olfactory receptors of the nose to the brain.

It is necessary to investigate the fear circuit by administering manganese by various routes. The intracerebral ventricular injection is less toxic as it delivers a minimal amount of manganese, but it is an effective way to deliver sufficient manganese to the brain (23). When manganese is injected into the ventricle, it is difficult for manganese to diffuse evenly over the entire brain area, because it usually accumulates strongly near the cannula. Careful manipulation of ventricular injection is required to reduce the inter-subject difference of manganese brain absorption. Alternatively, there is a method of administering manganese directly to a specific part of the brain (24). In this case, manganese can be used as an activity marker and tracer. It is possible to investigate the region where manganese is activated through synapses. The degree of absorption of the site adjacent to the administration reflects the degree of activation of a specific brain area. There is also a method of very slowly releasing manganese subcutaneously by using an osmotic pump (25). In this case, there is an advantage that there is little toxicity even when the same amount as one dose is administered. However, since it is administered for a long time, the time for manganese to be absorbed into the brain is extended and it is difficult to compare the behavior or conditions of a specific period. Instead, manganese is absorbed evenly throughout the brain, which is an advantage. By administering manganese through several routes, investigating neural circuits under different experimental conditions, and integrating the results will facilitate understanding the overall circuit. On the other hand, we used a fast spin-echo sequence to capture manganese-enhanced signals in this study. However, if we use more optimized sequences or measure T1 map, we may detect manganese signals better and find extensive neural circuits involved in fear conditioning.

In this experiment, we administered manganese directly to the rat nose and monitored activated brain regions for fear conditioning using MEMRI. We found the essential brain regions involved in fear and stress. The study shows that the MEMRI technique is an effective way to study the neural circuits involved in learning, emotion, and stress response combined with behavioral tests.

Acknowledgments

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