Reduction of Freezing Behavior by Acupuncture Stimulation at HT7 in Contextual Fear-Conditioned Mice

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Objective: Acupuncture at acupoint Shenmen (HT7) is used for the treatment of neuropsychological disorders, such as anxiety disorders, in oriental medicine. In the present study, to elucidate the influence of HT7-acupuncture on the reduction of fear memory, we examined freezing responses in contextual fear-conditioned mice with or without acupuncture treatment at HT7.

Materials and Methods: Adult male C57BL/6J mice were subjected to electric foot-shock, which represents a common animal model of post-traumatic stress disorder (PTSD), in a foot-shock cage. The mice were then immediately returned to their home cages and randomly divided into two groups, an HT7 and a sham group. In the HT7 group, 30 seconds of bilateral acupuncture at HT7 was applied for 7 consecutive days. We then placed the mice from both groups either in their home cages or in the foot-shock cage and observed their freezing behavior for 2 minutes.

Results: In the home cage, HT7 and sham mice displayed almost no freezing responses. In the foot-shock cage, in contrast, the sham group showed freezing responses for 51.3 ± 16.7% in 2 minutes, indicating that in these mice, fear memory was strongly maintained even after 1 week. Surprisingly, the freezing responses of the HT7 group mice in the foot-shock cage were significantly lower, at 25.7 ± 14.9% in 2 minutes.

Conclusions: These findings suggest that acupuncture treatment at HT7 might be effective in reducing fear memories in PTSD.

Key words: acupuncture, anxiolytic effects, fear, freezing behavior, Shenmen (HT7)

Introduction

Acupuncture is a treatment that stimulates a specific point, an acupoint, on the body by inserting an acupuncture needle. Recently, it has been reported that acupuncture treatment has positive effects on neuropsychiatric diseases such as anxiety disorders1), autism2), schizophrenia3) and Alzheimer’s disease (AD) 3), the underlying mechanisms remain however unclear.

Acupuncture is usually applied at multiple acupoints simultaneously. For the treatment of anxiety, for example, acupuncture stimulation has been applied to Baihui (GV20), Neiguang (PC6), Shenmen (HT7), Sanyinjiao (SP6), and Yintang (EX-HN3) 4) 5). Acupuncture treatment at PC6, HT7, and Taichong (LR3) has been reported to be effective for alleviating anxiety in patients with chronic anxiety, while it had no effect on general interventions 6). Acupuncture stimulation at Sishen-cong (EX-HN1), Shenting (GV24), EX-HN3, HT7, PC6, and Taixi (KD3) was found to not only reduce anxiety but also to improve memory functions in healthy undergraduate students 7).

Recently, research on acupuncture has also been conducted in the laboratory animals such as mice and rats 8) 9). Although there had been a lack of consensus among researchers regarding the location
of acupoints in the laboratory animals such as mice and rats, the definition of exact position is currently adopted by the positional method, which transposed the acupuncture points of human beings onto animal body. In addition, it has been suggested that the stimulation of acupoints including PC6 and HT7 in human and mouse displays similar effects.

Among acupoints, stimulation at HT7 has been suggested to play an important role in treating neuropsychological impairments. Although single-point acupuncture at HT7 has not been reported to show obvious anxiolytic effects in male undergraduate students exposed to the Trier Social Stress Test, several studies using animal models have clearly demonstrated anxiolytic effects even after single stimulation at HT7.

Acupuncture stimulation at HT7 in the maternal separation (MS) rat model, which causes vulnerability to stress and development of depression-like syndrome, resulted in the reduction of anxiety-related behaviors and the modulation of hypothalamic-pituitary-adrenal (HPA) system activation. The rat pups exposed to the MS paradigm also showed the reduction of depression-like behaviors and the increase in the expression of serotonin transporter in the prefrontal cortex, as well as the decrease in plasma levels of corticosterone, a biological stress marker.

Acupuncture treatment has also been found to be effective in the treatment of post-traumatic stress disorder (PTSD). Stimulation at multiple acupoints including HT7 quickly and permanently reduced fear responses to the traumatic memories and related cues in patients with PTSD. In addition, the effect of acupuncture at HT7 or HT8 has been examined with a rat model of single-prolonged stress (SPS), one of the PTSD animal models, and found to lead to a significant antidepressant effect in the forced swimming test.

However, the neural mechanisms underlying effects of acupuncture treatment for PTSD are poorly understood, and it is thus indispensable to establish a simple experimental model for the investigation of these mechanisms. In the present study, we chose fear-conditioning by electric foot-shock and a single acupoint, HT7, as the PTSD animal model and as the target of acupuncture treatment, respectively. Electric foot-shock models have been widely used in preclinical studies, since they are easily repeatable and suitable for the analysis of cognitive functions, with freezing responses reflecting re-experiencing of the traumatic event. In addition, accumulating studies have revealed anatomical and physiological neural mechanisms underlying fear memories induced by electric foot-shock.

Methods

1. Animals

All procedures involving animals were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the experiments were approved by the Committees for Animal Care and Use at Kyushu University (Med Kyo 17038) and at Juntendo University (310129). Thirty-six adult male C57BL/6 mice (8-12 weeks old; Japan SLC, Hamamatsu, Japan) were used in the present study. Mice were housed under a 12-hour light-dark cycle. To avoid disruptions in the mice’s sleep cycle, all behavioral experiments were conducted during the dark cycle. All efforts were made to minimize animal suffering and the number of animals used.

2. Fear conditioning with electric foot-shock

Mice were singly housed in their home cages for 7 days, and then moved to the foot-shock cage (MSK-001; Muromachi Kikai, Tokyo, Japan). Four minutes after habituation in the foot-shock cage, random electric foot-shock was applied to each mouse (0.5 mA, 2 seconds; SG-1000E; Melquest, Toyama, Japan). The mice were kept in the foot-shock cage for an additional 2 minutes, and then returned to their home cages.

3. Acupuncture treatment at HT7

The mice were first randomly divided into two groups of 18, an HT7 and a sham groups (see Figure-1A for the complete process and group design). The acupoint HT7, also called Shenmen, is located at the lateral side of the point where the tendon of the flexor carpi ulnaris muscle and the palmar wrist crease cross (see Figure-1B). The HT7 group received acupuncture treatment at HT7 daily for 1 week as follows. After disinfecting the acupoint HT7 with an alcohol cotton swab, an acupuncture needle (ϕ0.18 mm × 15 mm; 044-0133;
Seirin, Shizuoka, Japan) was inserted at a depth of 2–3 mm into the acupoint HT7. The acupoint HT7 was stimulated on both sides for 30 seconds, with the needle being rotated twice per second. In the sham group, the left and right forelimbs were fixed for 30 seconds, but no needle stimulation was applied. The mice were kept in their home cages, except during the acupuncture stimulation.

4. Freezing behavior analysis

Mouse behavior was recorded with a video camera (iVIS HF G20; Canon, Tokyo, Japan), for 2 minutes each time, in the home cage after the foot-shock, in the home cage after the first acupuncture treatment, in the home cage after the seventh acupuncture treatment, and finally in the home cage or foot-shock cage for the freezing test. When the mouse showed no movement for 1.5 seconds or more except for breathing, this was counted as a freezing response. The video file was opened with the software ImageJ (ver. 1.52a; National Institutes of Health), and the freezing reaction was analyzed manually according to the above criteria. We calculated the ratio of freezing time relative to the 2 minutes of video recording.

The movie file name was encrypted according to a random number table, and the key was stored by a third party. The analysis was performed after all experiments were completed.

5. Statistical analysis

Multiple comparisons were performed using non-parametric tests, that is, the Kruskal–Wallis test followed by the Steel–Dwass multiple comparison test, using EZR software (version 1.38; Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (version 3.5.3; R Development Core Team).
Results

1. Acupuncture treatment at HT7 in contextual fear-conditioned mice

After single housing in the home cage, the mice were transferred to the foot-shock cage and received electric foot-shock for 2 seconds (Figure-1A). The mice showed a jump-up avoidance response during the foot-shock. Then, the mice were divided into two groups: acupuncture treatment at HT7 was applied to an HT7 group but not to a sham group. The acupuncture needle was inserted into the acupoint HT7 on both hands, which is located on the lateral side of the intersection of the flexor carpi ulnaris muscle tendon and the palmar wrist crease (Figure-1B). After 7 days of acupuncture treatment, both the HT7 and the sham groups were further subdivided, and freezing responses were examined one by one in the home or foot-shock cage. There were no obvious behavioral abnormalities due to the acupuncture treatment.

2. The effect of HT7-acupuncture on freezing responses

Mouse behavior was recorded for 2 minutes each time, and the percentage of time the mice showed freezing behavior was calculated. In the home cage, after the electric foot-shock (Figure-2A), first acupuncture treatment (Figure-2B), and seventh acupuncture treatment (Figure-2C), almost no freezing responses occurred in any of the groups (0-6.3%), indicating that the acupuncture treatment did not elicit freezing responses.

On the freezing test, while the sham group in the home cage (Sham–HC) showed almost no freezing response (1.6 ± 1.5%), the sham group in the foot-shock cage (Sham–FC) displayed freezing responses for about half the time (51.3 ± 16.7%; Figure-2D). This indicates that the HT7-FC mice maintained their memory of the electric foot-shock in the foot-shock cage, at least partially, and that it was not erased. It has been reported that acupuncture treatment can improve memory impairments, and clinical studies have demonstrated that acupuncture treatment in patients with mild-to-moderate AD improved mood and cognitive functions. A functional magnetic resonance imaging study also revealed that multiple brain regions related to cognitive functions, such as memory function, were activated by acupuncture stimulation including HT7 in patients with AD. In addition, stimulation at HT7 improved cognitive function and increased glucose metabolism in the neocortex and hippocampus in a rat model of AD, and acupuncture stimulation at multiple acupoints including HT7 in healthy undergraduate students resulted in the improvement of memory function as well as the reduction of anxiety. It can thus be assumed that, in the present study, memory function was at least maintained and HT7-acupuncture affected the mice’s feeling of fear.

Discussion

In the present study, we show that acupuncture treatment at HT7 is effective in reducing freezing responses of fear-conditioned mice. Since freezing behavior has been considered to reflect re-experiencing of a traumatic event, acupuncture stimulation at HT7 can be assumed to be effective in reducing the fear memory related to the electric foot-shock in the foot-shock cage. However, fear memory is important in that it helps to escape from dangerous situations and thus plays a critical role in the survival of an individual. It should therefore be carefully considered whether the reduction in fear memory found here is due to the reduction in fear emotion or due to the impairment of memory function.

During the freezing test (Figure-2D), freezing times in the HT7-FC group decreased to about half, compared to the Sham-FC group, but they were significantly higher than freezing times in both home cage groups, the Sham–HC and the HT7–HC groups. This indicates that the HT7-FC mice maintained their memory of the electric foot-shock in the foot-shock cage, at least partially, and that it was not erased. It has been reported that acupuncture treatment can improve memory impairments, and clinical studies have demonstrated that acupuncture treatment in patients with mild-to-moderate AD improved mood and cognitive functions. A functional magnetic resonance imaging study also revealed that multiple brain regions related to cognitive functions, such as memory function, were activated by acupuncture stimulation including HT7 in patients with AD. In addition, stimulation at HT7 improved cognitive function and increased glucose metabolism in the neocortex and hippocampus in a rat model of AD, and acupuncture stimulation at multiple acupoints including HT7 in healthy undergraduate students resulted in the improvement of memory function as well as the reduction of anxiety. It can thus be assumed that, in the present study, memory function was at least maintained and HT7-acupuncture affected the mice’s feeling of fear.

Effects of acupuncture treatment have also been examined using an SPS animal model, another
model of PTSD. Acupuncture at HT7 or HT8 alleviated depression-like behaviors in the forced swimming test, whereas stimulation at HT8 but not HT7 induced anxiolytic effects in the open field and elevated plus maze tests. In addition, acupuncture at HT8 increased the expression of synaptic proteins such as post-synaptic density 95 (PSD95) and synapsin I (Syn1) in the hippocampus via the signaling pathway of mammalian target of rapamycin. It was also reported that electroacupuncture at GV20, GV24, and Shenshu (BL23) significantly improved PTSD-like symptoms, increased the expression levels of PSD95, Syn1, and growth-associated protein 43, and up-regulated the long-term potentiation in the hippocampus of rats exposed to SPS. Various acupoints including HT7 have shown positive effects on animal models of PTSD; it remains to be clarified at the neural network level, however, how various different types of acupuncture stimulation other than HT7 in PTSD

Figure 2
A: Freezing responses in the home cage just after electric foot-shock. The behavior of the mice was recorded for 2 minutes and analyzed for freezing response times. The numbers indicate the mean values and standard deviations (n=9 mice in each group). Each dot represents freezing time in 2 minutes of individual mouse. B: Freezing responses in the home cage after first acupuncture at HT7. C: Freezing responses in the home cage after seventh acupuncture at HT7. D: Freezing responses in the home cage or foot-shock cage. Statistical comparisons were performed using the Kruskal-Wallis test followed by the Steel-Dwass multiple comparison test, and p-values were shown.

FC, foot-shock cage; FS, foot-shock; HC, home cage.
models affect neural mechanisms. Furthermore, careful consideration must be given to whether rodents and humans are homologous to the acupoints\textsuperscript{10} and that acupuncture stimulation can cause nerve damage and other mechanisms such as the immune system.

The present study demonstrates a positive effect of single-point acupuncture at HT7 on a PTSD animal model induced by electric foot-shock. Studies using electric foot-shock models have led to remarkable progress in our understanding of the neural mechanisms underlying fear memory in PTSD\textsuperscript{22}. We therefore believe that our system, that is, acupuncture treatment at a single acupoint, HT7, for fear-conditioning induced by electric foot-shock, represents a useful model to study the neural substrates underlying acupuncture treatment for PTSD from both anatomical and physiological perspectives.

In conclusion, our findings show that acupuncture stimulation at HT7 reduces freezing responses in mice that were fear-conditioned with electric foot-shock. Electric foot-shock has been used as a model of PTSD, with the degree of retained fear memory inferred by the duration of the animal’s freezing behavior. Our findings thus suggest that HT7-acupuncture treatment might be effective in the treatment of PTSD. Since stimulation of acupoints other than HT7 has also shown improvements of PTSD-like symptoms, the influence of acupuncture stimulation at each acupoint on neural circuits should be further investigated in future studies.

**Conflicts of interest**

The authors declare no potential conflicts of interest.

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