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The effect of acupuncture on anxiety and neuropeptide Y expression in the basolateral amygdala of maternally separated rats

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Abstract

Recent studies have suggested that maternally deprived rats exhibit anxiogenic-like behavior when exposed to stress in later life. Neuropeptide Y (NPY) is involved in the regulation of various physiological functions such as the expression of anxiety. Female Wistar rat pups were separated from their mothers for 3 h daily from postnatal days 3 (P3) to 14 (P14). Acupuncture groups were treated with acupuncture at Shenmen (HT7) or Zusanli (ST36) on alternate days from P50 to P62. Their anxiety-like behavior was evaluated using an elevated plus-maze at P62, and then NPY immunohistochemistry in the basolateral amygdala (BLA) was performed. Rats exposed to maternal separation (MS) were less likely to explore the open arms of the plus-maze compared to control rats that were not exposed to MS. Among maternally separated groups, the percentage of time spent in the open arms was significantly increased in the HT7 acupuncture group, but not the ST36 acupuncture group, compared to MS group. In accordance with this behavior, the numbers of NPY-immunoreactive cells in the BLA were lower in the MS group compared to the control group. Among maternally separated groups, the numbers of NPY-immunoreactive cells in the BLA were significantly higher in the HT7 acupuncture group, but not higher in the ST36 acupuncture group, compared to MS group. These findings suggest that acupuncture treatment might reduce anxiety-like behavior in adult rats following maternal separation by modulating the NPY system in the amygdala.

Keywords: Acupuncture; Anxiety; Neuropeptide Y; Amygdala; Maternal separation

A large body of evidence indicates that exposure to early adverse life events in the form of childhood neglect and abuse can increase vulnerability to psychopathology in adulthood [11]. It has been suggested that maternal separation (MS) manipulation serves as a potential experimental model for psychiatric conditions such as depression or anxiety disorders [2,8]. Maternally separated adult offspring exhibit signs of increased anxiety in the open-field, plus-maze, auditory startle, and novelty-induced suppression of feeding tests [2,8,14,23].

Neuropeptide Y (NPY) is widely distributed throughout the central nervous system, with particularly high concentrations in the hypothalamus, and may be directly implicated in several stress-related pathological disorders and anxiety-related disorders [26]. The anxiolytic effects of NPY have been demonstrated in a wide range of pharmacologically validated animal models, including the conflict test, elevated plus maze test and fear-potentiated startle paradigm [1,6,7]. Based on experimental evidence in rodents, amygdalar NPY and NPY receptors play a critical regulatory role in the expression of anxiolytic behavior. Local infusion of NPY into the amygdala decreases anxiety-like behavior when activating either NPY1 or NPY5 receptors [19]. As many studies suggest that exogenous amygdalar NPY may be critical in anxiogenic action, it is likely that the anxiety-like behavior of maternally separated rats may reflect suppression of endogenous amygdala expression of NPY.

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Acupuncture has been shown to have therapeutic effects in several disorders that are induced by acute and chronic stress [16]. Recent clinical trials have demonstrated that acupuncture or auricular acupuncture reduce insomnia and anxiety in humans [20,22]. Previous studies showed that acupuncture at HT7, an acupuncture point that is used for mental disorders [21], enhanced locomotor activity and NPY expression in the hippocampus, and resulted in a significant increase in the number of BrdU-positive cells in the dentate gyrus of rats with depression-like disorder caused by MS [13,16]. Although the effects of acupuncture on MS-induced disorders, including depression, have been examined at a young age, little is known regarding possible therapeutic effects in other disorders, such as anxiety, in adults. Based on previous studies, it was thought possible that acupuncture at HT7 would enhance anxiolytic action by modulating NPY expression within the basolateral amygdala (BLA) of maternally separated rats.

The aim of the present study was therefore to investigate the effects of acupuncture on MS-induced anxiety in rats by evaluating performance in the elevated plus maze as a behavioral marker for anxiety-like behaviors, and to observe changes in NPY expression in the BLA in order to elucidate the possible mechanism of anxiolytic-like effects that we found.

Adult female rats were bred from Wistar females and males in our colony under constant temperature ($23 \pm 2\,^{\circ}C$) and lighting (12-h light:12-h dark cycle, lights on at 07:00 h) conditions. The day of birth was designated postnatal day 0 (P0). Pups were randomly redistributed among dams to eliminate any possible effects of genetic and prenatal factors. To reduce disruption of the mother–infant relationship, the animals were not handled until the time of deprivation (i.e., weaning). Litters were weaned on P21. On P28, females were marked, weighed, and placed in groups of two or three. As anxiety was more pronounced in females than in males in the MS group as compared to the control group, males were not included in this study to minimize sex-based variation [9]. Subsequent experiments were conducted on animals after P50. The experimental procedures were carried out according to the animal care guidelines of the NIH and the Korean Academy of Medical Sciences.

Pups were separated from their mothers for 3 h daily between 19:00 and 22:00 h from P3 to P14. MS consisted of removing the mother and then the pups from the home cage early in the light period. All of the pups from a given litter were placed together in a tub cage lined with bedding material for 3 h; the pups and then the mothers were then returned to their home cage. Control animals were returned to the foster dam immediately after group assignment (P3), and left undisturbed until weaning on P21 (Control, $n = 6$). On P50, the maternally separated rats were randomly assigned to one of three groups ($n = 18$): an experimental group that was subjected to MS (MS, $n = 6$), an acupuncture group that was subjected to MS and acupuncture at point Shenmen (HT7) (MS + HT7, $n = 6$), or an acupuncture group that was subjected to MS and acupuncture at point Zusanli (ST36) (MS + ST36, $n = 6$).

For acupuncture stimulation, stainless needles (0.2 mm in diameter) were inserted about 3 mm into the left and right side of the selected acupuncture points. The needles were twisted twice a second for 30 s and then removed. The stimulated areas correspond to acupuncture points in humans. HT7 is located at the end of the transverse crease of the ulnar wrist of the forepaw, and ST36 is near the knee joint of the hind limb, 2 mm lateral to the anterior tubercle of the tibia. The MS groups and the control group were both slightly immobilized for 30 s. All treatments were repeated on alternate days from P50 to P62.

The elevated plus-maze tests were used as a rodent model for human anxiety. This test is known to assess internal conflict between voluntary approach and withdrawal tendencies [18]. As this test is based on a natural fear of open and elevated spaces, the number of entries into open arms and the time spent on open arms are negatively correlated with the anxiety level of the subject. In addition to these parameters, other ethologically derived measures were scored, including the total number of open and closed arm entries, as a general measure of locomotor activity in the maze, so as to rule out any non-specific effects that might interfere with the interpretation of the data. The experimental apparatus consisted of a plus-formed maze that was elevated 50 cm above the ground. The four arms were each 40 cm long and 10 cm wide. Two opposing arms were enclosed by black wood walls 30 cm high (closed arms), while the other arms were devoid of walls (open arms). A total of 24 rats were tested individually on the plus-maze without any pretest handling in adulthood. Each rat was habituated to the testing room for at least 20 min. Each rat was placed in the center of the maze, after which the cumulative time spent on each arm and the numbers of entries into the open or closed arms were recorded during a 5-min test session at P62. The area inside the center portion (10 cm × 10 cm) was not considered. Entry by an individual into an arm was defined as commencing when the animal placed all four paws in that arm. The maze was cleaned with water after each rat had been tested. Exploration of the open arms was encouraged by testing under indirect dim light (2 × 60 W). Behavior in the maze was recorded via a video camera mounted on the ceiling above the center of the maze and relayed to a system that included a monitor, video tracking, motion-analysis, and a behavior-recognition system (S-MART, Spain). The data were recorded as time spent in open arms expressed as a percentage of total time spent in the arms.

For NPY immunohistochemistry, animals were anaesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with 100 ml of saline followed by 900 ml of a 4% solution of paraformaldehyde prepared in phosphate-buffered saline (PBS). The brains were removed, postfixed in the same fixative for 2–3 h at $4\,^{\circ}C$, placed overnight in PBS containing 20% sucrose at $4\,^{\circ}C$, and cut into 40-μm thick coronal sections through the hypothalamic areas. After

being washed twice with PBS, the sections were incubated overnight with rabbit anti-NPY anti-body (DiaSorin, Stillwater, MN, USA) at a dilution of 1:2000, then washed twice again in PBS and incubated for 1 h with biotinylated anti-rabbit antibody (1:200). After washing in PBS followed by incubation for 90 min in PBS containing avidin biotinylated horseradish peroxidase complex (1:100, Vector Laboratories, Burlingame, CA, USA), the sections were washed again in PBS. Sections were mounted on slides, dehydrated, and cover slipped for microscopic observation. For reproducible comparison among the experimental groups, measurements were performed at the same coordinates with respect to bregma according to the atlas of Paxinos and Watson: basolateral amygdaloid complex; bregma −2.56 mm [17]. The NPY-immunoreactive (ir) cells in each section were counted using a bright-field microscope (Olympus, Japan) and analyzed using an image analyzer (Optimas version 6.5, Media Cybernetics, MD, USA). Cell counts were expressed as the mean number of NPY-positive cells per unit area (mm^2). All quantification procedures were performed blind.

Results were expressed as mean ± S.E.M. of the total number of entries and the percentage of time spent on the open arms relative to the total time spent on the open and closed arms in the elevated plus-maze. Behavioral data were statistically analyzed by analysis of variance (ANOVA). The origin of significant effects was further examined by post hoc comparisons using the Tukey HSD technique. For immunocytochemical data, the mean numbers of NPY-ir neurons were calculated and analyzed by one-way ANOVA followed by the Tukey HSD technique. Significant differences among groups were defined as $P < 0.05$.

The behavioral data from the elevated plus-maze are illustrated in Fig. 1. Statistical analysis showed that the number of total entries in the elevated plus-maze did not show any significant differences among the four groups [$F(3, 23) = 0.837$, $P = 0.490$]. An ANOVA revealed a significant effect on the percentage of time in the open arms within groups through the trials [$F(3, 23) = 4.464$, $P < 0.05$]. Post hoc Tukey' HSD comparison indicated that the MS group spent a significantly smaller percentage of time in the open arms than did the control group ($P < 0.05$). However, the HT7 acupuncture group spent a significantly greater time in the open arms than did the MS group ($P < 0.05$), while the ST36 acupuncture group did not statistically differ from the MS group ($P = 0.181$).

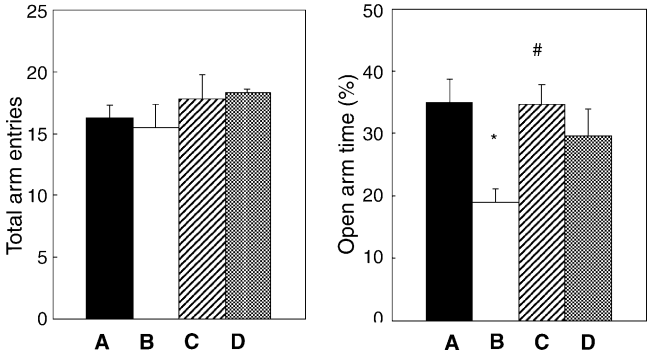

Fig. 1. Performance of the Control (A), MS (B), MS + HT7 (C), and MS + ST36 (D) groups in the elevated plus-maze. Total open and closed arm entries in the elevated plus-maze during a 5-min test were measured (mean ± S.E.M.). The percentage of time spent in open arms (open/open + closed) during the 5-min test was also measured (mean ± S.E.M.). Total open and closed arm entries did not show any significant differences among groups (left side). Rats in the MS group spent less time in open arms and had lower scores than those in the control group. The scores of rats in the MS + HT7 group were higher than those of rats in the MS group (right side), but the scores of rats in the MS + ST36 group were not. $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. MS group.

Photomicrographs of NPY-ir neurons in the BLA are shown in Fig. 2. An ANOVA revealed a significant effect on NPY-ir neurons in the BLA within groups [$F(3, 23) = 8.934$, $P < 0.01$]. Post hoc Tukey' HSD comparison indicated that the MS group had significantly fewer NPY-ir neurons in the BLA than did the control group ($P < 0.05$). However, the HT7 acupuncture group had significantly more NPY-ir neurons in the BLA than did the MS group ($P < 0.01$), while the ST36 acupuncture group did not statistically differ from the MS group ($P = 0.837$) (Fig. 3).

Evidences suggest that repeated MS for 3 h daily during the "stress hypo-responsive period" between P3 and P14 of life, considered a chronic intermittent postnatal stress, produces anxiogenic behavior, as indicated by a reduction in the amount of time spent in the open arms of an elevated plus-maze [8,15,23]. On the other hand, other researchers have observed no changes in the open field or elevated plus-maze tests in adult rats that were subjected to a 24-h maternal deprivation protocol, implying unchanged anxiety levels [12]. These discrepancies may be owing to many factors, including the maternal deprivation protocol, the age of the offspring, and differences in the control groups used. Our experiments, however, demonstrated that rats exposed to neonatal MS showed increased anxiety-like behavior. These results are in agreement with previous reports, which demonstrated that MS increased fearfulness and reduced exploration [2,14,15,23]. In addition, the number of total entries was also analyzed as a measure of locomotor activity. The results indicated no significant difference among the four groups following treatment. Thus, the anxiety-like behavioral effects observed cannot be attributed to changes in locomotion.

NPY is a 36-amino acid peptide that has a role in the regulation of various basic physiological functions and is abundant throughout the peripheral and central nervous systems [5]. The role of NPY in anxiety-like behavior has been firmly established in experimental animals. Exogenous administration of NPY produces anti-anxiety actions in all models tested, including ethologically derived paradigms (such as the elevated plus-maze and the social interaction test), models based on fear suppression of behavior (including non-operant punished drinking and the operant food-reinforced paradigm), and fear potentiated startle [1,6,7]. As very little research has been conducted into the role that NPY plays in the effect of acupuncture on anxiety-like behavior, we conducted the present study to evaluate the role of NPY in

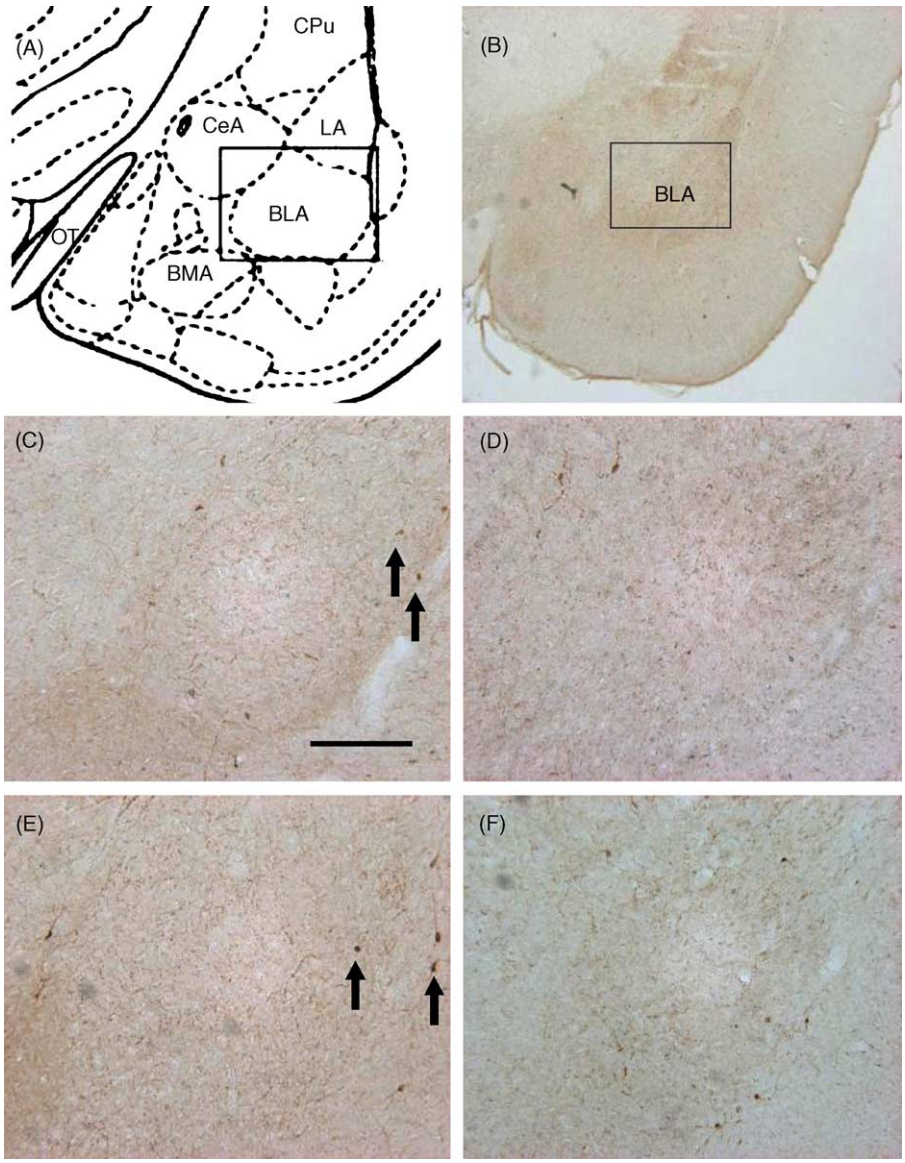

Fig. 2. (A) Schematic diagram, adapted from the atlas of Paxinos and Watson, showing the area of the amygdala in which NPY-ir was quantified. (B) Representative photomicrograph of a coronal section showing the region of the BLA where NPY-ir neurons in the BLA were quantified. Representative photomicrographs showing the area of the amygdala in which NPY-ir was quantified in Control (C), MS (D), MS + HT7 (E), and MS + ST36 (F) groups. LA, lateral nucleus; BLA, basolateral amygdaloid nucleus, anterior part; BMA, basomedial amygdaloid nucleus, anterior part; CPu, caudate putamen; OT, optic tract. Scale bar = 200 μm.

the effect of acupuncture on anxiety in maternally separated rats.

The amygdala is a limbic brain region that is involved in the control of emotional behavior [4]. The BLA, a subdivision of the larger basolateral complex, plays a crucial role in decision-making and in adaptive response selection to affective stimuli [10]. A local infusion of NPY into the BLA was recently found to decrease anxiety-like behavior while activating either NPY1 or NPY5 receptors [19]. Furthermore, the number of NPY-ir neurons was significantly greater in the basolateral nucleus of inbred Roman high-avoidance rats, and thyroxine treated animals displayed reduced anxiety-like behavior as well as an increase in the number of NPY-ir neurons in the basolateral complex of the amygdala [24,25]. In accordance with previous reports, our results also showed that neonatal MS decreased the number of NPY-ir neurons in the BLA.

Acupuncture treatment has been reported to be valuable for some categories of anxious human patients with insomnia [20]. Auricular acupuncture at the “relaxation” point also decreased anxiety levels in a population of healthy volunteers [22]. In the present study, acupuncture at HT7 in maternally separated rats resulted in a significant reduction in anxiety-like behaviors and increased the number of NPY expressing cells in the BLA, compared to MS group. These anxiolytic effects of acupuncture are in accordance with several hu-

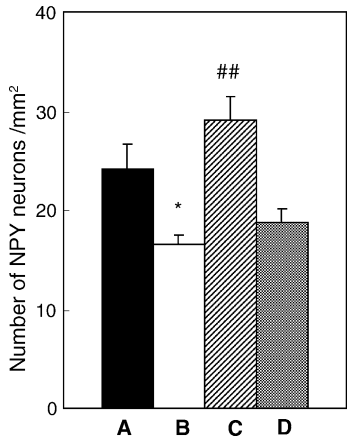

Fig. 3. Quantitative analysis of NPY in the BLA of the Control (A), MS (B), MS + HT7 (C), and MS + ST36 (D) groups. Data are represented as the mean ± S.E.M. of NPY-ir neurons. The numbers of NPY-ir neurons in the BLA were significantly lower in the MS group than in the control group, whereas the numbers of NPY-ir neurons in the BLA were significantly higher in the MS + HT7 group, but not in the MS + ST36 group, compared to the MS group. $^{*}P < 0.05$ vs. control group, $^{\#\#}P < 0.01$ vs. MS group.

man studies. However, unlike the HT7 acupuncture group, the ST36 acupuncture group showed no significant changes as compared to the MS group. These results can be explained by the fact that each acupuncture point exhibits different effects. Acupuncture point HT7 has been used to treat mental disorders, while acupuncture point ST36 has been used to regulate gastrointestinal function, relieve pain, and modulate nicotine addiction [3].

To summarize, the present results demonstrate that in maternally separated rats, acupuncture at HT7 decreased anxiety-like behavior and increased the number of NPY-immunoreactive cells in the BLA. These findings suggest that acupuncture can improve mood-related disorders such as anxiety.

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