

Acupuncture at ST₃₆ prevents chronic stress-induced increases in neuropeptide Y in rat

Ladan Eshkevari¹, Rupert Egan², Dylan Phillips³, Jason Tilan¹, Elissa Carney², Nabil Azzam⁴, Hakima Amri⁵ and Susan E Mulroney²

¹Department of Nursing, School of Nursing and Health Studies; ²Department of Pharmacology & Physiology; ³Department of Human Science, School of Nursing and Health Studies; ⁴Department of Neuroscience; ⁵Department of Biochemistry and Cell and Molecular Biology, Georgetown University Medical Center, Washington, DC 20007, USA

Corresponding author: Ladan Eshkevari, School of Nursing and Health Studies, 421 St Mary's Hall, 4000 Reservoir Road, NW, Washington, DC 20007, USA. Email: eshkevl@georgetown.edu

Abstract

Chronic stress, as seen in post-traumatic stress disorder, can exacerbate existing diseases. Electroacupuncture (EA) has been proposed to treat chronic stress, although information on its efficacy or mechanism(s) of action is limited. While many factors contribute to the chronic stress response, the sympathetic peptide, neuropeptide Y (NPY), has been shown to be elevated in chronic stress and is hypothesized to contribute to the physiological stress response. Our objective was to determine if EA at acupuncture point stomach 36 (ST₃₆) is effective in mitigating cold stress-induced increase in NPY in rats. Both pretreatment and concomitant treatment with EA ST₃₆ effectively suppressed peripheral and central NPY after 14 d of cold stress ($P < 0.05$). The effect was specific, as NPY in Sham-EA rats was not different than observed in stress-only rats. Additionally, the effect of EA ST₃₆ was long-lasting, as NPY levels remained suppressed despite early cessation of EA ST₃₆, while exposure to cold stress was continued. In the paraventricular nucleus (PVN), it was notable that changes in NPY mirrored plasma NPY levels, and that the significant elevation in PVN Y1 receptor observed with stress was also prevented with EA ST₃₆. The findings indicate that EA ST₃₆ is effective in preventing one of the sympathetic pathways stimulated during chronic stress, and thus may be a useful adjunct therapy in stress-related disorders.

Keywords: acupuncture, NPY, chronic stress, paraventricular nucleus

Experimental Biology and Medicine 2012; **237**: 18–23. DOI: 10.1258/ebm.2011.011224

Introduction

Chronic stress has been linked with cardiovascular disease and obesity,^{1–10} and has been shown to depress immune function, possibly influencing the course of cancer and infections.^{11–13} Effectively blocking the stress pathways remains a challenge, and new therapies are continually being sought: acupuncture is a possible candidate. Acupuncture is the most common treatment modality in traditional Chinese medicine (TCM),¹⁴ and electroacupuncture (EA), the electric stimulation of acupuncture needles, has been utilized by the Chinese for treating numerous diseases. According to the World Health Organization, acupuncture is useful as adjunct therapy in more than 50 disorders including chronic stress;¹⁵ however, the mechanisms by which EA acts are unclear.

Traditionally, a common TCM acupoint, Zusanli (ST₃₆), has been used in patients for a variety of conditions including stress.¹⁴ However, reports on mechanisms of EA actions during stress are limited, and existing studies have focused

on acute stress rather than chronic stress responses.^{16–18} Experiments during acute stress show that EA ST₃₆ blocks chronic stimulation of the hypothalamic–pituitary–adrenal axis (HPA), and thus might reduce the physiological effects of the acute stress. Indeed, Sun *et al.*¹⁷ found that EA ST₃₆ in their rat model was effective at reducing corticosterone concentrations, and modulated the expression of nitric oxide synthase_{1–3}, possibly playing a role in protecting gastric mucosa from acute cold-induced stomach ulcers. Such studies, while intriguing, do not address the potential for EA in chronic stress states or its mechanism of action. Our broad hypothesis is that EA acts through several stress pathways including the HPA and sympathetic nervous system (SNS). Initially, we examined the effect of ST₃₆ on one component of the SNS, neuropeptide Y (NPY).

NPY is primarily synthesized and released by sympathetic neurons both centrally and peripherally; it is also released from platelets in many species.^{19–21} Importantly, plasma NPY is elevated in response to stress, and is

proposed to contribute to the deleterious physiological effects of chronic stress.^{22,23} Various stress models have provided evidence that NPY also increases in brain regions such as the amygdala and the paraventricular nucleus (PVN),^{24,25} as well as in sympathetic ganglia.^{26–28} These stress-induced effects have been mainly attributed to the action of NPY at the NPY Y1 receptor (Y1R).²⁵ Using a previously established model of non-habituating chronic cold stress,²⁹ we examined the effect of EA ST₃₆ on central and peripheral NPY in rats.

Methods

Animals

All animal experiments were approved by the Georgetown University Animal Care and Use Committee. Adult male, 11–12-week-old, Sprague-Dawley rats (Harlan Laboratories, Inc, Dublin, VA, USA) weighing 290–420 g were included in this study. The rats were received with 23G round-tip indwelling jugular vein catheters (JVCs). On receipt, they were weighed and randomly assigned to one of either four or five groups depending on the experiment. Animals were assigned to one or two control groups; a stress-only group; a Sham-EA group that received the stress treatment and non-TCM EA; and finally a TCM-EA group.

Rats were housed one per cage in a controlled environment at a constant temperature (23°C) and maintained in a 12-h light/dark cycle with free access to water and regular rat chow. Catheter care was conducted on the day after the animals had arrived, and then again on days one and seven of the experiment. Maintenance consisted of drawing back on the catheter with a 1-cm³ sterile syringe utilizing a 23G blunt tip sterile stainless steel needle (Small Parts, Inc, Seattle, WA, USA), followed by instilling 0.1 mL of the Harlan recommended lock solution: 5 mL 100% sterile glycerin (Fisher Scientific, Pittsburgh, PA, USA) with 5 mL 1000 U/mL heparin sodium.

All animals were acclimated similarly for four days before the onset of interventions. Acclimation included three minutes of touch and placing the animal twice a day in a cotton sock, which was subsequently used in the study to briefly hold the animal during the acupuncture procedures. The control animals received no further treatments once the experiment was begun.

Acupuncture

Sham-EA needling

Acupuncture treatment for the Sham-EA group consisted of placing the animal into the sock that had been utilized for acclimation. Sterile, stainless steel, acupuncture needles measuring 34G (0.22 mm) and 1 inch (25 mm) (Millennia, Shanghai, China) were inserted bilaterally into a randomly designated non-TCM acupuncture point on the back, 2 cm lateral to the tail region.

TCM acupuncture needling

The rats in the TCM EA group received acupuncture treatments utilizing the same sock and needles as described

above. However, in this cohort, the location of the needles was at TCM point Zusanli (Stomach, ST₃₆) on bilateral hind legs of the animal. These points were identified using rat mapping for TCM acupuncture (kindly provided by the laboratory of Dr Lixing Lao, University of Maryland School of Medicine, Baltimore, MD, USA).

EA treatments

Once the acupuncture needles described above were inserted bilaterally, they were attached to electrodes. Animals were then singly placed in new cages with electrodes threaded through the cage lid. Electrodes were then attached to a power source (Model AWQ-104L, purchased from UPC Medical Supplies, St Gabriel, CA, USA) and the points were stimulated for 20 min at a frequency of 10 Hz with 2 mA output, and a pulse width of 0.1 s for 20 min, based on previous findings.^{30,31} The animals did not receive food or water for the duration treatment. All acupuncture treatments occurred between 09:00 and 12:00 depending on the experiment, and were administered either 30 min before the stressor or 30 min after the animals had acclimated to ambient room temperature, in their home cages.

Two EA ST₃₆ paradigms were assessed: *PRE* (EA prior to initiation of stress) – to determine whether pretreatment with EA could diminish the stress response, animals received 20 min of either Sham or EA ST₃₆ treatment for five days prior to 10 d of cold stress. Control ($n = 7$), Sham-EA ($n = 10$), Stress ($n = 7$), EA ST₃₆ ($n = 10$); and *POST* (EA begins following exposure to stress) – to determine whether EA treatment after initial exposure to stress effectively diminishes the stress response, rats received Sham or EA ST₃₆ treatment for 20 min following one hour of cold stress for 14 d. Control ($n = 7$), Sham-EA ($n = 13$), Stress ($n = 7$), EA ST₃₆ ($n = 13$). In this model, animals received one-hour cold stress, were then returned to their home cage for 30 min at room temperature, followed by 20 min of Sham or EA ST₃₆. To assess whether the effects of EA ST₃₆ were long lasting, we ceased treatment in four animals from the *POST* Sham-EA and EA ST₃₆ groups four days before the experiment ended, while continuing with stress.

Stress

Animals were randomly assigned to treatment groups. Cold stress was carried out as previously described.³² Briefly, animals were placed in cages with 1 cm deep crushed ice for one hour per day for 14 d. Following cold stress, animals were returned to their home cage.

NPY enzyme-linked immunosorbent assay

Blood was obtained via the JVCs using 23G blunt stainless steel needles (Small Parts Inc) in 3-mL sterile syringes. In general, 1.3 mL of whole blood was collected in 1.5-mL ethylenediaminetetraacetic acid tubes and centrifuged at 4°C at 400g for six minutes to in order to separate out the platelet contribution of NPY, followed by centrifugation at 4°C at 10,000g for two minutes in order to isolate the NPY fraction presumably from sympathetic nerves.³³ Plasma was extracted using 200 mg C-18 sep-columns (Y-1000;

Bachem, San Carlos, CA, USA). NPY plasma levels were measured by enzyme-linked immunosorbent assay (S-1145; Bachem), according to the manufacturer's procedure.

Immunohistochemistry analysis

Animals were anesthetized using 30–50 mg/kg pentobarbital (intraperitoneally). Brain hemispheres were harvested and cut in half for immunohistochemistry and quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. Tissues were post-fixed with 10% buffered formalin, paraffin-embedded and prepared as 5 μ m-thick sagittal sections. Slides were deparaffinized with xylene and rehydrated through a graded alcohol series, starting with 100% EtOH and ending with 60% EtOH. Slides were immersed in 10 mmol/L citrate buffer (pH 6.0) with 0.05% Tween at 98°C for 20 min. Horseradish peroxidase (HRP)-conjugated antirabbit secondary antibody (Envision-Plus; DAKO USA, Carpinteria, CA, USA) was applied, and the HRP was detected using DAB chromagen (DAKO USA). Similarly treated serial sections with the primary antibody omitted were used as negative controls.

Sections were visualized with the CRi Nuance FX microscope and pictures were taken with the CRi Nuance v2.6.0 camera (Caliper Life Sciences, Hopkinton, MA, USA). The PVN was identified using rat brain map,³⁴ in consultation with a neuroanatomist (Dr Nabil Azzam), and semiquantitatively analyzed for positive NPY staining via the MDS Analytical Technologies Metamorph v7.5.5.0 (Sunnyvale, CA, USA).

Quantitative realtime RT-PCR

Total RNA was isolated from resected brain PVN using the previously described phenol–chloroform extraction

method.³⁵ One microgram of RNA per sample was used for cDNA synthesis via the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Realtime RT-PCR was done using the ICycler iQ Detection System (Bio-Rad). The PCR reactions were carried out using TaqMan Universal PCR Master Mix and predesigned primers and fluorescein-labeled probes (Applied Biosystems, Foster City, CA, USA). The primers were as follows. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5' CCTTCATTGACCTCAACTAC 3', 5' GGAAGGCCATGCCC AGTGAGC 3'; NPY: 5' CAGAGGC GCCCAGAGCAG 3', 5' CAGCCCCATTCGTTTGTTACC 3'; Y1R: 5' CTCTTGCTTATGGRGATGTGA 3', 5' CTGGAAGT TTTTGTTTCAGGAAYCCA 3'. The results were calculated by the comparative threshold method using GAPDH as an endogenous reference gene, according to the Applied Biosystems ABI PRISM 7700 User Bulletin no. 2.

Statistical analysis

Data are presented as mean $\pm$ SEM and analyzed by one-way analysis of variance with Tukey multiple *t*-test or Kruskal–Wallis post-test depending on the sample size to compare between treatment groups using GraphPad Prism v4 (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered statistically significant for the indicated *n* per group.

Results

Effect of EA on plasma NPY in chronically stressed rats

Control values for platelet-poor plasma NPY were comparable (within normal levels) in both PRE and POST groups on the pretreatment day (day 1, Figure 1a, left and right panels). By day 14, NPY was significantly higher in the stress-only and Sham-EA groups compared with controls

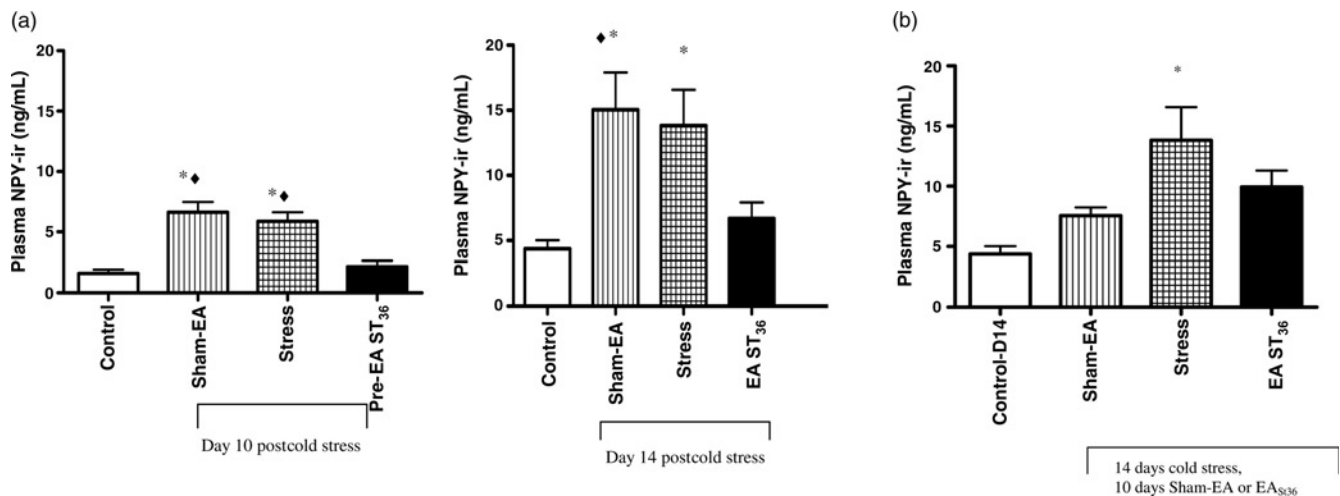


Figure 1 Effect of EA given before and after stress on plasma NPY in chronically stressed rats. (a) Plasma NPY levels on days 1 (pre-treatment), and 14, respectively. PRE-treatment (left panel); POST-treatment (right panel). By day 14, plasma NPY levels in the stress + Sham-EA, and the stress-only groups were significantly higher than the control animals ($P < 0.05$) in both PRE and POST groups. In sharp contrast, EA ST₃₆ prevented this increase and NPY was not different from control plasma NPY levels, indicating that EA ST₃₆ is effective when given either before or after the stressor. (b) Long-term effects of EA ST₃₆ on plasma NPY in chronically stressed rats. The plasma NPY levels in the stress-only animals were significantly elevated when compared with control animals ($P < 0.05$). NPY in the stress + Sham-EA and stress + EA ST₃₆ animals was not significantly different from controls, despite cessation of EA on day 10. The interesting reduction in NPY in the stress + Sham-EA animals four days after cessation of EA may relate to the addition of acute pain/stress-induced NPY caused by the Sham-EA over days 1–10. NPY in stress + EA ST₃₆ animals were not significantly different from controls, despite cessation of EA on day 10, suggesting that the effects of EA ST₃₆ last several days. NPY, neuropeptide Y; EA, electroacupuncture

(Figure 1a). In contrast, EA at ST₃₆ prevented the stress-induced increase in NPY. Indeed, NPY levels in the EA ST₃₆ rats were not different from controls, but were significantly lower than the Sham-EA and stress-only animals (Figure 1a). These findings were consistent in both the PRE and POST EA ST₃₆ animals, indicating that EA ST₃₆ is effective in preventing the rise in plasma NPY, whether given following or prior to the stress.

To investigate if the effects of acupuncture on the plasma NPY levels are transient or long lasting, we also analyzed NPY levels in animals where EA treatments were stopped during the last four days of cold stress in POST animals. At day 14, as previously seen, NPY in the stress-only animals was significantly greater than seen in the control, non-stressed group ($P < 0.05$), while EA ST₃₆ was not different from controls (Figure 1b). The Sham-EA group was also not different from controls; this was different from the NPY levels observed in the animals receiving Sham-EA for the entire experiment (Figure 1b).

Effects of EA on NPY Y1R expression in the PVN

Central NPY immunoreactivity was examined by immunohistochemistry in tissue from the PVN. There were significant increases in NPY in the PVN of stress-only and Sham-EA groups (Figure 2a), and the effect of EA ST₃₆ to prevent the increase in NPY protein was similar to effects observed in PVN NPY mRNA (Figure 2b). The changes in gene expression mirrored the effects observed in the plasma (Figure 1). However, these changes in the brain NPY were different than those observed in the adrenal glands, where NPY mRNA expression was not different between the groups (data not shown).

As the Y1R is considered the predominant receptor for NPY action in the PVN during chronic stress,¹⁷ Y1R mRNA expression was examined in the PVN from control

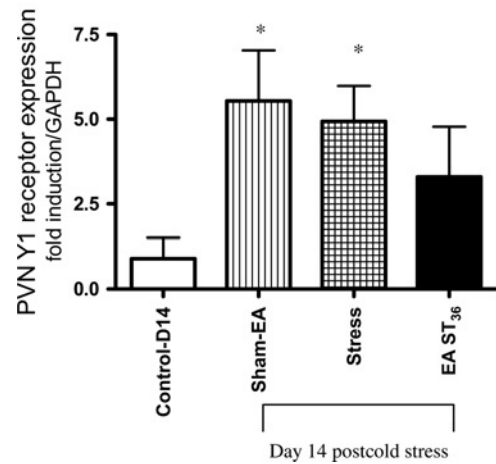


Figure 3 Effect of EA on NPY Y1 receptor mRNA expression in the PVN of chronically stressed rats. NPY Y1 receptor expression (normalized by GAPDH) was significantly higher in the stress + Sham-EA and stress-only animals when compared with the controls ($^*P < 0.05$). EA ST₃₆ prevented the stress-induced increase in NPY Y1 receptor. NPY, neuropeptide Y; EA, electroacupuncture; PVN, paraventricular nucleus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

and stressed animals. There was a significant induction of Y1R mRNA in the PVN of stress-only animals ($P < 0.05$ from controls), which was prevented by EA ST₃₆ (Figure 3). Y1R mRNA was also significantly increased by Sham-EA ($P < 0.05$ from controls), further confirming the specificity of the EA ST₃₆. The Y2 receptor did not vary between the groups, supporting the findings that the Y1R effect was specific (data not shown).

Discussion

The current study demonstrates that EA specifically at ST₃₆ is effective in preventing the chronic stress-induced increase

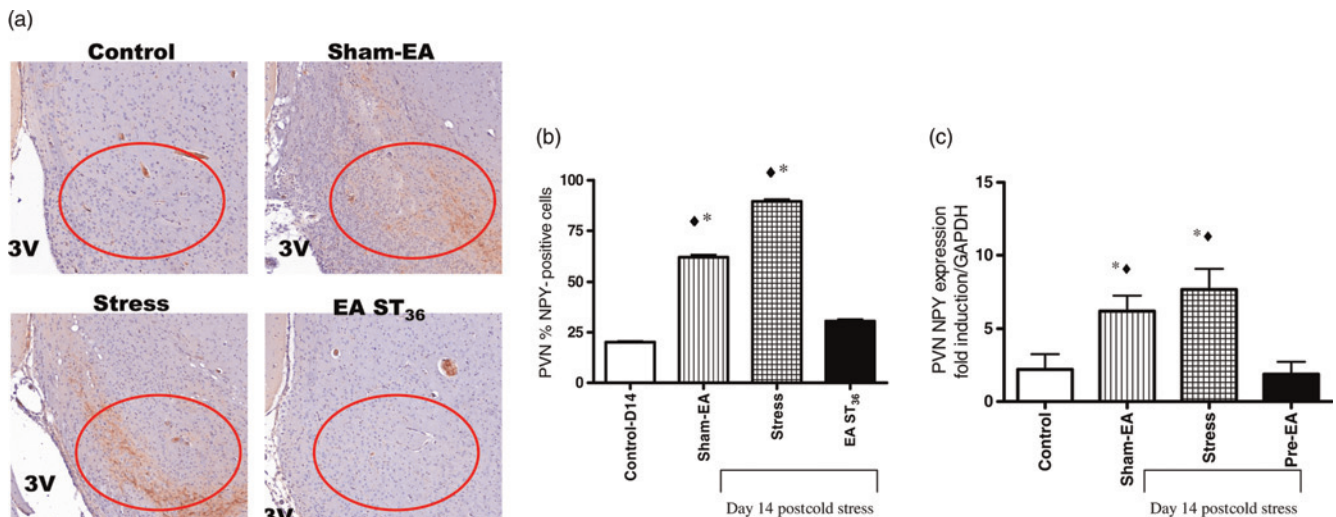


Figure 2 Effect of EA on NPY protein and message in the PVN of chronically stressed rats. (a, b) NPY immunohistochemistry in the PVN. NPY immunoreactivity was significantly higher in the stress + Sham-EA and stress-only animals when compared with the control group ($^*P < 0.0001$). EA ST₃₆ prevented this increase. (c) NPY mRNA expression in the PVN. NPY mRNA (normalized by GAPDH) was also significantly higher in stress and stress + Sham-EA than either control or stress + EA ST₃₆ animals ($^*P < 0.05$). NPY, neuropeptide Y; EA, electroacupuncture; PVN, paraventricular nucleus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

in circulating and central NPY. In separate experiments, the cold stress-induced three-fold increases in plasma NPY were blocked by both pre- and post-treatment with EA at ST₃₆ (but not with Sham-EA). In addition, this effect appears to be long lasting, providing suppression of NPY for four days after EA ceased. Finally, these changes in peripheral NPY were also observed in NPY protein in the PVN of the hypothalamus. This ability to block NPY has elucidated a potential mechanism of action for acupuncture's efficacy in treatment of chronic stress. We have therefore added to the literature by reporting for the first time that both PRE- and POST- EA ST₃₆ are effective in preventing the chronic stress-induced increases in plasma and PVN NPY, and that the effect may be long lasting.

The plasma NPY stress-induced response was noted at 14 d, but not at seven days (data not shown), supporting the role of NPY as contributing to the chronic response to stress. These findings support the previous work showing that cold stress increases NPY in plasma.^{36–38} EA has been demonstrated to decrease NPY in other physiological stress conditions, including diabetes and chronic food restriction. Lee *et al.*³⁹ found that acupuncture at ST₃₆ decreased NPY expression in the hypothalamus of rats with streptozotocin diabetes, reducing diabetic hyperphagia. Tian *et al.*⁴⁰ reported that EA decreased expression of gastric ghrelin and hypothalamic NPY in chronic food-restricted rats when compared with their controls. Our studies extend these reports by showing that EA ST₃₆ is also effective at blocking central and plasma NPY during chronic stress. In addition, we determined that when EA was withdrawn during cold stress, NPY continued to be low in EA ST₃₆ rats. No other studies have demonstrated these potentially long-lasting effects of EA ST₃₆.

Another important aspect of this work is the central effects of EA ST₃₆ on NPY in the PVN, which mirrored that of the NPY in the plasma. The chronically stressed animals had significantly higher Y1R mRNA expression when compared with the control animals; the increase was prevented by EA ST₃₆. These central effects of EA ST₃₆ make the overall effects more compelling and relevant, since changes in brain NPY have also been postulated in humans during times of stress.⁴¹

It is unclear at what point the EA ST₃₆ may block the central and peripheral response to release NPY during chronic stress; however, we speculate that EA ST₃₆ may act to block one or more pathways. Indeed, other studies in our lab indicate that EA ST₃₆ is also effective in blocking the HPA (unpublished results), which may also modulate the NPY response. This, in addition to the potential effect of EA ST₃₆ to block the SNS, may comprise an important mechanism to suppress HPA and sympathetic outflow in response to chronic stressors.

In our model of chronic cold stress, peripheral and central NPY levels were significantly increased, which is in accordance with other reports.^{17–20} Thus, one of the mechanisms by which EA reduces chronic stress may be by suppressing the NPY pathway and reducing the stress-induced increase in central Y1R; however, in the absence of the protein data, conclusions on the receptors have to be viewed with caution. This study suggests that EA ST₃₆ is effective in

preventing one of the sympathetic pathways stimulated during chronic stress, and thus may be a useful adjunct therapy in treating chronic stress-related disorders.

Author contributions: LE was the primary researcher. This work was based on her ideas and expertise in stress and acupuncture; all the acupuncture was conducted by her; and most of the manuscript was written by her. RE and DP carried out the RT-PCR along with JT who also contributed with his expertise in NPY. They assisted in writing the methods section for RT-PCR. EC assisted with the immunohistochemistry and helped in writing this section of the paper. NA assisted with the location of the PVN; as a neuroanatomist he contributed by examining the brain slices and determining the correct locations of the various nuclei, especially the PVN which was of interest in this work. Part of the work was conducted in HA's physical lab. SEM oversaw the project, assisted greatly with writing and editing of the manuscript and contributed to the study design.

ACKNOWLEDGEMENTS

The research was funded by the American Association of Nurse Anesthetists (AANA) doctoral fellowship award to LE and NIH/NCCAM grant no. K07-AT001193–02 NIH/NCCAM to HA.

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(Received June 23, 2011, Accepted September 2, 2011)