Acupuncture blocks cold stress-induced increases in the hypothalamus–pituitary–adrenal axis in the rat

Ladan Eshkevari, Eva Permaul¹ and Susan E Mulroney²

School of Nursing and Health Studies, Georgetown University Medical Center, 421 St Mary's Hall, 3700 Reservoir Road NW, Washington, District of Columbia 20007, USA

Departments of ¹Histopathology and ²Pharmacology and Physiology, Georgetown University Medical Center, Washington, District of Columbia 20007, USA

Correspondence should be addressed to L Eshkevari

Email
eshkevl@georgetown.edu

Abstract

Electroacupuncture (EA) is used to treat chronic stress; however, its mechanism(s) of action in allaying stress remains unclear. The interplay of stress hormones of the hypothalamus–pituitary–adrenal axis (HPA) and the sympathetic nervous system (SNS) is critical in the stress response. Our objective was to determine whether EA at acupoint, stomach 36 (EA St₃₆) is effective in preventing chronic cold stress-induced increased hormone levels in the rat by examining four groups of animals, three of which were exposed to cold and one of which was a non-treatment control group. Before exposure to the cold, two groups were treated with either EA St₃₆, or Sham-EA, before 10 days of cold stress. The EA St₃₆ animals demonstrated a significant decrease in peripheral HP hormones (ACTH and CORT) compared with stress animals ($P < 0.05$). These effects were specific; rats receiving Sham-EA had elevation of these hormones, similar to the stress-only animals. These effects were mirrored centrally in the brain; CRH levels were significantly ($P < 0.05$) reduced in EA St₃₆ animals compared with the other animals. Finally, EA effect on peripheral and adrenal SNS hormones (norepinephrine (NE) and neuropeptide Y (NPY) respectively) was examined, with no significant difference noted in adrenal tyrosine hydroxylase or circulating NE in any of the groups. However, EA St₃₆ was effective in preventing stress-induced elevation in adrenal Npy mRNA. These results indicate that EA St₃₆ blocks the chronic stress-induced elevations in the HPA and the sympathetic NPY pathway, which may be a mechanism for its specific stress-allaying effects.

Key Words

- stress
- HPA
- SNS
- acupuncture

Journal of Endocrinology
(2013) 217, 95–104

Introduction

The classic response to chronic stress consists of an elegant, concerted interplay of two important pathways, the sympathetic nervous system (SNS) and the hypothalamus–pituitary–adrenal axis (HPA). The chronic activation of these stress pathways can lead to maladaptive homeostatic conditions causing symptoms or diseases

such as depression, anxiety, and obesity, which may have a direct impact on cardiovascular disorders and hypertension (Szczechanska-Sadowska *et al.* 2010, Tamashiro 2011, Tamashiro *et al.* 2011, Tran *et al.* 2011, McEwen 2012, McEwen *et al.* 2012). The initial response to stress consists of an increase in the central expression of numerous

catecholamines and peptides in the sympathetic ganglia such as norepinephrine (NE) and neuropeptide Y (NPY) (Nankova *et al.* 1996, Bernet *et al.* 1998). NPY and NE have been found to cross talk with the HPA in the CNS causing release of CRH. However, in chronic stress while NE levels return to normal, NPY levels remain elevated, thereby playing a modulating role in NE release in chronic stress (Renshaw *et al.* 2000, Cavadas *et al.* 2001, Heilig 2004). As such, NPY plays a cost-saving role for NE, so it can be reserved for more acute responses, during times of chronic stress. Concomitantly, the increase in hypothalamic CRH stimulates the release of ACTH from the pituitary gland. This leads to a subsequent release of corticosteroids as well as NE and epinephrine from the adrenal glands.

Indeed, the HPA and SNS are anatomically and functionally unified; during stress, they interact both in the periphery and in the CNS (Li *et al.* 2000, Jacobson 2005, Dimitrov *et al.* 2007, Kakui & Kitamura 2007). The hypothalamic paraventricular nucleus (PVN) is a major site for direct neuronal NPY and NE input to CRH cell bodies (Suda *et al.* 1993, Li *et al.* 2000). In several rodent models of chronic stress, such as cold stress, the increase in PVN NPY leads to stimulation of CRH synthesis and subsequent increase in serum corticosterone (CORT) levels (Jacobson 2005, Kakui & Kitamura 2007). It has also been observed that injection of NPY into the PVN increases circulating ACTH and CORT, while ACTH has been demonstrated to exert modulating influences on adrenal NPY (Suda *et al.* 1993, Hinson *et al.* 1998).

Given the negative physiological impact of chronic stress, blocking the stress-induced effects of the SNS and HPA may provide a much needed protection against stress-related disorders. Acupuncture has been widely used for over 2500 years across Asia and has most recently gained popularity in Western cultures. Traditional Chinese Medicine (TCM) practitioners have traditionally used electroacupuncture (EA) in the treatment of stress-related disorders; however, there has been little evidence to support its efficacy as an anxiolytic. Two studies have shown that acupuncture can block acute stress-induced increases in the HPA axis (Han *et al.* 1999, Yang *et al.* 2002). Although there is limited information on chronic stress, we have recently reported that EA at acupoint Zusanli, stomach 36 (St₃₆) can ameliorate chronic cold stress-induced increases in central (PVN) and peripheral (plasma) NPY in rats, providing one potential mechanism of action of acupuncture on reducing stress (Eshkevvari *et al.* 2012).

The objective of the current study was to determine whether application of EA at St₃₆ (EA St₃₆) during chronic cold stress blocks the stress-induced increases in the major

hormones of the HPA and how changes may be related to the adrenal SNS response. This model of chronic stress has been well established by others in our department; Kuo *et al.* (2007) used this model to study the role of NPY in the relationship between stress and obesity (Kuo *et al.* 2007). We therefore used this chronic stress/pain paradigm to examine PVN CRH, adrenal NE and NPY, and circulating CORT and ACTH in sham and EA St₃₆ rats after 10 days of cold stress. We chose St₃₆ because it is a potent point on the human meridian point system according to TCM. It is used in many instances in TCM, including stress.

Materials and methods

Animals

The animal experiments in this study were approved by the Georgetown University Animal Care and Use Committee (GUACUC), in compliance with National Institute of Health guidelines. A total of 34 adult male Sprague Dawley rats weighing 290–420 g, with indwelling jugular catheters, were received from Harlan Laboratories, Inc. (Dublin, VA, USA). The rats were randomly assigned to four groups. Groups 1 and 2 were controls ($n=7$ per group): group 1 received no treatments (Control group), while group 2 was the stress-only (Stress) group in which the animals were exposed to 10 days of cold stress. The two experimental groups ($n=10$ per group) consisted of a sham EA group (Sham-EA), which received 4 days of pretreatment with EA at a sham point 5 mm away from the rat tail, bilaterally with no stress, followed by Sham-EA for the next 10 days immediately preceding exposure to the cold stress; the fourth group was the experimental EA group, which was pre-treated with EA St₃₆ for 4 days before the cold stress and continued to receive EA St₃₆ immediately before exposure to the cold stress for 10 additional days. EA St₃₆ was specifically used, not only because of its efficacy to manage stress according to TCM but also due to its location (Fig. 1). St₃₆ is located 0.5 cm below fibular head of the hind leg in rat bilaterally and is easily acupunctured while the rat remains conscious. It was important to conduct the acupuncture on non-anesthetized animals in our study, given the confounding known effects of anesthetics on stress hormones.

The rats were housed one per cage, to protect the jugular catheters, and the room was kept at a constant temperature of 23 °C. The jugular catheters were checked on the day of arrival. Catheter care that consisted of ensuring patency with a 1 cc sterile syringe and a 23 G blunt tip sterile stainless steel needle (Small Parts, Inc.,

Seattle, WA, USA), followed by injection of 0.1 ml of the Harlan recommended lock solution: 5 ml 100% sterile glycerin (Fisher Scientific, Pittsburg, PA, USA) with 5 ml 1000 U/ml heparin sodium, was performed on days 1 and 7 of the experiment. The animals were maintained in a 12 h light:12 h darkness cycle with free access to food and water. All animals were familiarized with the experiment personnel for 4 days before the onset of the experiments by petting the animals and allowing them to crawl into a cotton sock, which was later used in the protocol to momentarily hold the animal in place while inserting acupuncture needles. The control animals (Control) received no further treatments once the experiment was begun, while the stress control group received only cold stress.

Acupuncture

The acupuncture protocol consisted of placing the animal; head first, into the cotton sock previously used in the acclimation period. Once the animals were comfortably secured in the sock, sterile, stainless steel, single-use acupuncture needles measuring 34 G (0.22 mm) and 1 inch (25 mm) (Millennia, Shanghai, China) were quickly inserted either in the Sham point or the EA St₃₆ point, bilaterally. The electrodes were wrapped quickly around the needle and were taped in place to the needle and then to the rat. The electrodes were subsequently attached to an EA machine (Model AWQ-104L, UPC Medical Supplies, San Gabriel, CA, USA). The animals were placed in a single cage on bedding material and did not receive food or water for the duration of the treatment, which lasted for 20 min. The points were stimulated at a frequency of 10 Hz with 2 mA output, and a pulse width of 0.1 s, based on other rat models of acupuncture treatment (Lao *et al.* 2004, Zhang *et al.* 2005). The uniformity of the electrical output was achieved using an oscilloscope that measured the EA machine's outputs on all rats and adjusted to achieve the desired output. All acupuncture treatments occurred in the morning between 0900 and 1100 h and were administered 30 min before the initiation of the cold stress treatments, and animals were observed closely to ensure that needles were not displaced.

EA St₃₆ EA needling The rats in the EA St₃₆ group received EA treatments as described earlier. The location of the needles for EA St₃₆ is on bilateral hind legs of the animal. These points were identified using rat mapping for TCM acupuncture. We chose St₃₆ as the TCM acupoint of interest as it is considered one of the most potent and commonly used points in TCM practice. Additionally,

previous rodent stress studies have successfully demonstrated that St₃₆ can modulate stress pathways (Lim *et al.* 2003, Sun *et al.* 2005, Eshkeviri *et al.* 2012).

Sham-EA needling EA treatment for the sham group was the same as described earlier, except the points selected were non-acupuncture map points on the back of the animal, 2 cm lateral to the tail region. This point was used as the sham point as there are no known acupuncture points near them and they were out of reach of the animals.

Stress

On day 4 of the experiment, cold stress, which entailed placing the animal in 1 cm deep crushed ice (2–5 °C) for 1 h daily, was carried out as described previously (Zukowska-Grojec 1995). The stress, Sham-EA and EA St₃₆ animals received this daily 1-h cold exposure for 10 days. The animals in the Sham-EA and EA St₃₆ were placed in the cold stress cages 30 min after they had received their acupuncture treatments. Following the cold stress, animals were returned to their home cages and allowed to rest.

Blood sampling

Blood samples were collected on day 1 (before initiation of any treatments), day 7, and day 14 of the experiment via the indwelling jugular catheters using a 1 cc sterile syringe. Following blood collection, the sterile heparin lock solution was replaced and animals were returned to their cages. On days 7 and 14 of the protocol, the animals were exposed to the acupuncture and cold stress treatments and allowed to acclimate to room temperature (RT) for 1 h before bloods were drawn between 1100 and 1300 h. The blood sampling occurred between these times taking into account the diurnal nature and circadian fluctuations of ACTH and CORT. Plasma was collected in 1.5 ml EDTA tubes, centrifuged at 4 °C at 6708 g for 2 min, and the supernatant plasma was stored at –80 °C. Serum was collected in 1.5 ml low retention tubes, placed on a rack at RT for 15 min, and centrifuged at RT for 5 min at 5000 r.p.m. The supernatant serum samples were collected and stored at –80 °C.

Analysis of ACTH, CORT, and NE by ELISA

Each ELISA was performed in accordance with the manufacturer's recommended protocols. ACTH ELISA was performed using an ELISA kit (Cat #S-1185; Bachem,

San Carlos, CA, USA – specificity of 100% for rat ACTH with a typical sensitivity of 0.26 ng/ml). This kit required an initial extraction step that consisted of equilibrating a 200 mg C-18 sep-column with 1 ml 100% acetonitrile, followed by 1% trifluoroacetic acid (HPLC grade) 3 ml, three times, and centrifuged with a cold vacuum centrifuge once it was buffered. The collected residue was rehydrated with equal volume (150 μ l) of assay buffer, and used in the assay. CORT was determined using an ELISA kit (Cat #DSL-10-81100 with a specificity of 100% for rat CORT and a typical sensitivity of 1.6 ng/ml) from Diagnostic Systems Laboratories (Webster, TX, USA). NE ELISA kit (Cat #BA E-5200; Rocky Mountain Diagnostics, Colorado Springs, CO, USA – specificity of 100% for rat NE with a typical sensitivity of 0.2 ng/ml) was used to measure NE.

The optical density from each assay was read on a standard ELISA plate reader set at 450 nm.

Immunohistochemistry

Immunohistochemical staining for CRH was performed on formalin-fixed paraffin-embedded 5 μ m sections of rat brain PVN. As previously published, by Eshkevari *et al.* (2012), the PVN was located from dorso-ventral coordinate of 8.0 mm from Bregma and medio-lateral coordinate of 0.7 mm. The sections were deparaffinized with xylenes and rehydrated through a graded alcohol series, starting with 100% EtOH and ending with 70% EtOH, followed by rinsing in water and TBS with 0.05% Tween. For antigen retrieval, slides were immersed in 10 mM citrate buffer (pH 6.0) with 0.05% Tween at 98 $^{\circ}$ C for 20 min and allowed to cool for 20 min at RT. After blocking with 3% hydrogen peroxidase and 10% normal goat serum, slides were incubated with a primary antibody, CRH

Figure 1

(A and B) Effect of EA given before and concomitantly with chronic cold stress on HPA hormones. (A) Plasma ACTH levels: on day 7, all three experimental groups had significantly higher ACTH levels than the control group ($*P < 0.05$). However, by day 14, plasma ACTH levels in the stress-only and the stress + Sham-EA animals were significantly higher than the control animals ($*P < 0.05$). In sharp contrast, EA St₃₆ prevented this increase when compared with the stress-only animals ($#P < 0.05$). Furthermore, there was no significant difference between the ACTH plasma levels of the ST₃₆ animal compared with the controls. (B) Serum CORT levels: The serum CORT levels in the stress-only and Sham-EA animals were again significantly elevated when compared with control animals ($*P < 0.05$). EA St₃₆ prevented this elevation when compared with the stress-only and stress + Sham-EA serum CORT levels ($#P < 0.05$). EA St₃₆ had effectively prevented the stress-induced increases in serum CORT to levels similar to the control animals. (C) Plasma NE levels: there were no significant differences noted in the plasma NE levels of the four study groups.

(1:2000, Abcam ab8901, Cambridge, MA, USA) for 1 h at RT. HRP-conjugated anti-rabbit secondary antibody (Dako Envision-Plus, San Antonio, TX, USA) was applied for 30 min, and the HRP was detected using DAB chromagen (Dako). Slides were counterstained with hematoxylin (Harris modified hematoxylin; Fisher Scientific) at a 1:17 dilution for 2 min at RT, blued in 1% ammonium hydroxide for 1 min at RT, dehydrated, and mounted with Acrymount. Negative controls consisted of similarly treated consecutive brain sections with the omission of the primary antibody. Slides were visualized using the CRi Nuance FX microscope (Caliper Life Sciences, Hopkinton, MA, USA). The PVN was identified referring to the rat brain map (Paxinos & Watson 1998). Images (10 $\times$) were saved using the CRi Nuance v2.6.0 camera and were semi-quantitatively analyzed for positive CRH staining by the MDS Analytical Technologies Metamorph v7.5.5.0 (Sunnyvale, CA, USA).

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was used to determine PVN *Crh* mRNA expression as well as adrenal tyrosine hydroxylase (TH) and *Npy* mRNA expression. Briefly, total RNA was isolated from resected brain paraventricular nuclei using the previously described phenol-chloroform extraction method (Chomczynski & Sacchi 1987). One microgram of RNA per sample was used for cDNA synthesis via the iScript cDNA synthesis kit (Bio-Rad). Real-time RT-PCR was done using ICycler iQ Detection System (Bio-Rad). The PCRs were carried out using TaqMan Universal PCR Master Mix and predesigned primers and fluorescein-labeled probes (Applied Biosystems). GAPDH: 5'-CCTTCATTGACCTCAACTAC-3', 5'-GGAAGGCCATGC-CAGTGAGC-3'; CRH: 5'-CAA TCG AGC TGT CAA GAG AGC-3', 5'-GGA AGA AAT CCA AGG GCT GAG-3'; β -actin: 5'-GTG ACG TCC GTA AA-3', 5'-CTC AGG AGG AGC AAT GAT CTC-3'; TH: 5'-GCC ATG AGC TGT TGG GAC AT-3', 5'-CCC CAG AGA TGC AAG TCC AAT-3'; and NPY: 5'-CAG AGG CGC CCA GAG CAG-3', 5'-CAG CCC CAT TCG TTT GTT ACC-3'. The results were calculated by the comparative cycle threshold (CT) method using GAPDH for the brain, and β -actin for the adrenals as the endogenous reference genes, per the Applied Biosystems ABI PRISM 7700 User Bulletin #2.

Statistical analysis

Data were analyzed using GraphPad Prism v4 (GraphPad Software, La Jolla, CA, USA) and are presented as mean $\pm$ S.E.M. We used one-way ANOVA with Tukey multiple *t*-test or

Kruskal-Wallis post-test depending on the sample size to compare between treatment groups. $P < 0.05$ was considered statistically significant for the indicated sample size per group.

Results

Effect of EA St₃₆ on plasma ACTH and serum CORT in chronically stressed rats

There were no significant variances in any parameters after 7 days of cold stress among the experimental groups, with ACTH and CORT being elevated similarly in all three cold stress-exposed groups (Fig. 1A). However, after 10 days of cold stress, ACTH and CORT levels in stress-only and Sham-EA animals were significantly ($P < 0.05$) increased compared with the control group (Fig. 1A and B). In marked contrast, 4 days of pre-treatment with EA at acupuncture point St₃₆ (which continued for 10 days with concomitant exposure to cold stress), ameliorated the effects of cold stress: ACTH and CORT levels in stressed EA St₃₆ rats were not different from those seen in control animals and were also significantly lower than those of the stress-only and Sham-EA groups ($P < 0.05$) (Fig. 1A and B).

Effect of EA St₃₆ on plasma NE in chronically stressed rats

After 10 days of chronic cold stress, there were no significant differences in the plasma NE levels between any groups (Fig. 1C), which was not unexpected, considering changes in circulating NE may be related to more acute stress situations.

Effects of EA St₃₆ on PVN CRH

Figure 2 illustrates PVN *Crh* mRNA expression. Chronic exposure to cold stress resulted in significant increases in CRH message in stress-only rats and Sham-EA rats when compared with control animals ($P < 0.05$). Although there was no significant difference between EA St₃₆ and control CRH expression, PVN CRH in the EA St₃₆ group was also not significantly lower than the other two stress groups. (Fig. 2). A representative immunohistochemistry (IHC) identifying CRH protein in the different groups is shown in Fig. 3A. Figure 3B shows the average number of CRH-positive cells from each group. Stress significantly elevated CRH protein in the PVN compared with control animals ($P < 0.01$). Sham-EA did not prevent the increase; by contrast, CRH protein in EA St₃₆ rats was not

significantly different from unstressed controls, consistent with its effects on *Crh* mRNA, but was significantly ($P < 0.05$) lower than the stress-only and Sham-EA animals.

Effects of EA St₃₆ on adrenal NPY and TH

To start identifying how chronic stress-related changes in NE and NPY might relate to the HPA response, we studied the effects of cold stress on the adrenal medullary NPY and TH message (an important enzyme in the synthesis pathway of the catecholamines). We found that chronic cold stress had a significant impact on adrenal *Npy* mRNA expression in raising transcription activity when compared with the non-stressed control group ($P < 0.05$; Fig. 4A). EA St₃₆ prevented this significant ($P < 0.05$) increase in message, and levels were similar to that of the control animals. Again, the Sham-EA had failed to prevent the stress-induced increase in NPY (Fig. 4A).

Unlike the stress-induced changes observed in *Npy* mRNA, there was no change in TH mRNA levels in any of the experimental groups (Fig. 4B). This may again relate to the fact that NE plays a more important role in the acute response to stress while NPY exerts a more chronic effect on modulating NE and maintaining homeostasis in the face of chronic stress.

Figure 2

Effect of EA on CRH message in the PVN of chronically stressed rats. *Crh* mRNA (normalized by GAPDH) was significantly higher in the stress-only and stress+Sham-EA animals when compared with the control animals ($*P < 0.05$). Conversely, this was not observed when evaluating the *Crh* mRNA levels of the EA St₃₆ group to that of the control animals.

Figure 3

(A and B) Effect of EA on CRH protein expression in the PVN of chronically stressed rats. CRH immunoreactivity was significantly higher in the stress-only and the stress+Sham-EA animals when compared with the control group ($*P < 0.01$). EA St₃₆ prevented this increase ($*P < 0.05$). 3V, 3rd ventricle.

Discussion

Our findings indicate that EA St₃₆, beginning 4 days before induction of cold stress, can prevent the chronic stress-induced increases in the HPA, as well as SNS-related adrenal NPY. This effect was specific to St₃₆, as Sham-EA was not different to responses in stress-only animals. These findings indicate that the mechanism of action of EA St₃₆ involves blocking the HPA at (or before) the level of

Figure 4

(A and B) Effect of EA on adrenal NPY and TH expression. (A) Adrenal NPY levels: adrenal NPY levels of the stress-only and stress + Sham-EA animals were significantly higher than those of the control animals (* $P < 0.05$). By contrast, EA St₃₆ prevented this increase (* $P < 0.05$) such that Npy mRNA was similar to that of the control animals. (B) Adrenal TH expression: there were no significant variations noted in the adrenal TH expression when comparing groups.

PVN CRH. This supports our initial report of the efficacy of EA St₃₆ in preventing chronic cold stress-induced increases in central and peripheral NPY (Eshkevvari *et al.* 2012) and extends the information by reporting for the first time that EA ameliorates the effects of chronic stress on the HPA. These findings support targeted EA as therapeutic intervention in chronic stress conditions.

The phenomenon that EA could prevent elevations in ACTH levels in chronic stress was demonstrated in the late 1970s and early 1980s in rat and human models of chronic stress such as opioid withdrawal (Wen *et al.* 1978, Fung

et al. 1980). Our results support and extend these earlier findings in human models of stress. Indeed, pretreatment with EA at TCM point St₃₆ (Fig. 5) maintained ACTH at control animal levels, while those of the stress animals were significantly higher. In our model of pretreating animals, before exposure to chronic cold stress, with EA stimulation at St₃₆, we were able to ameliorate the cold stress effects. Furthermore, the pretreatment with EA St₃₆ maintained CORT at control levels, indicating that EA, specifically at St₃₆, was again effective at preventing the stress-induced increases in both HPA-related hormones in our model of chronic stress. Plasma ACTH levels are under tight negative-feedback control from the glucocorticoids, which are released in response to ACTH secretion. However, during chronic stress, CORT levels remain elevated engendering catabolic consequences that lead to breakdown of vital functions (Sapolsky *et al.* 2000). As EA St₃₆ (but not Sham-EA), prevented these stress-induced increases, it is possible that the deleterious effects of chronically high corticosteroids might be prevented in TCM EA-treated animals.

These findings are novel; there are currently no published data on the effects of acupuncture on peripheral ACTH and CORT levels in a chronic stress model such as the one we used. There have, however, been a few studies demonstrating the effects of EA on the HPA in acute stress, but the EA points used were not always specified. Han *et al.* (1999) discovered that in their rat acute stress model of tooth pulp stimulation, acupuncture had similar effects to those we found on the adrenocortical system. They also found that ACTH and CORT levels were decreased with EA at the large intestine meridian point 4 (LI4, a point commonly used in TCM for facial and dental pain) treatments after tooth pulp stimulation was begun (Han *et al.* 1999). Their animals had a similar rise in ACTH and CORT with the stressor, which was significantly lower by EA treatments. Interestingly, in another more recent study of the effects of EA on a painful, acute inflammation rat model, the investigators had contradictory findings: CORT levels were elevated by acupuncture treatments, which lead to better outcomes in reduction of inflammation and paw-withdrawal latency (Li *et al.* 2007). We believe the difference in our model and the aforementioned models may be due to varying pathways that are stimulated during EA procedures, such as to the arcuate nucleus and the periventricular hypothalamic nucleus, depending on the type and duration of stress, as well as the EA point used. Indeed, it is well established that there are various pain/stress ascending pathways, as well as various modulating descending pathways that could have played

Figure 5

Rat acupuncture point map. The circles mark the sham and acupuncture points St₃₆.

a role in acupuncture's specificity in acute vs chronic stress paradigms (Pan *et al.* 1997, Almeida *et al.* 2004, Gebhart 2004, Ogata *et al.* 2005, Saade *et al.* 2006). Indeed, new theories on stress point to a more specific response depending not only on the stressor but also on the ability of the organism to cope with the stressor and their perceptions, genetics, and environmental factors (Goldstein & Kopin 2007). Stressor-specific responses have been linked to numerous stressors that have been divided into four varying categories, which include i) physical stress such as cold and pain, such as the one in our model, ii) psychological stress, iii) social stress, and finally iv) homeostatic stressors such as hemorrhage or exercise (Kvetnansky *et al.* 2009). Stress can be further classified as chronic, as in our model, or acute, and again this may provide an explanation for the difference between our findings and those of the aforementioned study, which had used acute pain/inflammation as their model of stress.

In order to begin examining where in the HPA pathway EA may work, we investigated the central effects of EA St₃₆. Our finding that stimulation at acupoint EA St₃₆ prevents stress-induced increases in *Crh* mRNA (Fig. 2) mirrored the efficacy of stimulation on ACTH levels and was consistent with previous work that increased CRH is associated with a variety of stressors (Slawecki *et al.* 2005, Santibanez *et al.* 2006, Yanagita *et al.* 2007). However, currently, there are no reports on the effects of EA on stress-induced elevations of PVN CRH. We believe that as CRH plays an important role in initiation and integration of the stress response, the central effects of EA St₃₆ on CRH

PVN may explain the downstream effects we have observed in HPA hormone levels.

In addition to stimulating ACTH, CRH neurons from the PVN project to medullary and spinal cord autonomic neurons (49). Here, they regulate the adrenal and peripheral sympathoneural response to stress (Sawchenko & Swanson 1982, Swanson *et al.* 1986). Therefore, we also examined the effects of EA St₃₆ on plasma NE and adrenal NPY and TH activity, which are downstream of HP activation. Regulation of TH expression is a major mechanism by which NE and the adrenergic system respond to stress, and TH plays an integral role in adaptation to various stressors (Stone & McCarty 1983). In our model of chronic stress, we found no significant differences in plasma NE (Fig. 1C) or adrenal TH activity (Fig. 4B) between the groups. This finding is actually consistent with the fact that the NE response is primarily an acute effect to help regulate blood pressure and blood flow (Nankova & Sabban 1999, Nankova *et al.* 1999). The lack of change in plasma NE and adrenal TH levels in our groups is consistent with other investigators who have found that NE levels, although elevated at first, do indeed stabilize over time with exposure to the same stressor (Glavin 1985a, Glavin 1985b).

Acupuncture has also been used, with some success, in human acute stress models including acute mental stress (Middlekauff *et al.* 2002). However, there have been no recent studies on the effects of EA on the chronic stress-induced activation of the SNS. As part of the SNS response, stress is also a major activator of adrenal NPY, and various stressors including cold stress have been associated with increases in adrenal *Npy* mRNA (Renshaw & Hinson 2001). Our findings support and extend these previous studies, by demonstrating that adrenal *Npy* mRNA was significantly elevated after 14 days of stress, and EA St₃₆ prevented this increase in NPY expression. This suggests that NPY may modulate the chronic stress response in concert with the hormones of the HPA. Indeed, the stress-induced increase in adrenal NPY may result from central signals from either CRH or NPY, which we have previously shown to be elevated in the PVN of stressed rats (Eshkevvari *et al.* 2012). In either case, the ability of EA St₃₆ to block the increase in adrenal NPY could be via EA actions at the PVN.

We have demonstrated that treatment with EA at the specific point, St₃₆ starting 4 days before, and continuing through 10 days of chronic cold stress, can prevent increases in the central PVN hormone CRH expression, thereby preventing the stress-induced elevations in circulating ACTH and CORT levels. Furthermore, we have shown that peripherally, EA St₃₆ can influence

adrenal NPY levels, which may also contribute to its stress-ameliorating effects. An important area of future work would be to see if EA is effective when given after stresses have begun, which would have important implications for therapeutic intervention. Further studies will also need to be conducted to identify the potential effects of EA St₃₆ on the pain pathways activated in this model of chronic cold stress. Additionally, to further confirm our findings, it would be useful to block the pathways elucidated. We have demonstrated that EA St₃₆ is useful at reducing stress-induced elevations in HPA hormones. This may be of use therapeutically in stress-related disorders but warrants further investigation, perhaps in a human chronic stress model.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

The research was funded by the American Association of Nurse Anesthetists (AANA) doctoral fellowship award awarded to Dr L E, PhD.

Author contribution statement

L E, a nurse anesthetist, physiologist, and licensed acupuncturist was the main contributing author. The study was conducted based on her experience in treating chronic pain and stress patients; the idea to develop the study was thus generated. She wrote the bulk of the manuscript, with input from contributing authors below. E P, contributed to the set up of the IHC protocols and assisted with the Materials and methods section in the manuscript pertaining to IHC. S E M, provided guidance on study protocols and assisted with manuscript revisions and preparation.

Acknowledgements

The authors would like to thank the department of histopathology for their assistance with the IHC and Dr Nabil Azzam for his help with identifying the brain regions of interest. The authors would like to thank Dr Lixing Lao for his assistance in setting up our acupuncture protocols and providing us with the rat acupoint map (Li 2003). The authors would also like to thank Mr David Klemm, medical illustrator at Georgetown University, for his redrawing of the rat acupuncture points used in the study.

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Received in final form 30 January 2013

Accepted 4 February 2013

Accepted Preprint published online 4 February 2013