THE STRESS REDUCING EFFECTS OF ACUPUNCTURE
IN A RAT MODEL OF CHRONIC STRESS

A Dissertation
Submitted to the Faculty of the
Graduate School of Arts and Sciences
Of Georgetown University
In partial fulfillment of the requirements for the
Degree of
Doctor of Philosophy
In Physiology and Biophysics

By

Ladan Eshkevari, M.S. CRNA, L.Ac.

Washington, DC
November 19, 2009
ABSTRACT

There is a growing body of evidence that links chronic stress to the development of numerous disease processes including cardiovascular disease, cancer and obesity. Electro-acupuncture (EA) is a useful adjunct therapy in the treatment of many disorders including stress and anxiety. Although, EA is utilized worldwide as an anxiolytic and a stress reliever, its general mechanism of action, especially with regards to chronic stress pathways, has not yet been elucidated. Hence, the overarching hypothesis for this project was that EA allays the stress response, and that it does so by affecting the two classical stress pathways: the hypothalamic-pituitary-adrenal axis (HPA) and the sympathetic nervous system (SNS). We therefore investigated the possible role of EA in lowering both central and peripheral stress hormones associated with these pathways in a chronic-stress rat model. The HPA hormones investigated in our chronic stress paradigm were brain corticotrophin releasing hormone (CRH), plasma adrenocorticotropin hormone (ACTH), and serum corticosterone (CORT). The SNS response markers of interest were adrenal tyrosine hydroxylase (TH), an important enzyme in the synthesis of
norepinephrine (NE), plasma neuropeptide Y (NPY) and (NE). In our chronic
stress paradigm stress-induced elevations in plasma neurohormones
associated with the HPA and SNS were suppressed upon treatment with EA.
Although NE levels were unchanged in our model of chronic stress, EA was
effective at reducing adrenal mRNA expression levels of NPY and tyrosine
hydroxylase (TH). These findings were confirmed by the significant decrease of
mRNA expression and immunoreactivity of corticotropin releasing hormone
(CRH) and NPY, in the paraventricular nucleus (PVN). In some of the protocols
EA was also able to modulate NPY receptors Y1 and Y2 in the brain PVN.
Behavioral testing further confirmed the central effects of EA in allaying stress-
induced depression and anxiety in our stress paradigm. Findings from blocking
the HPA and SNS by RU-486 and propranolol, respectively, indicated that
although the SNS was modulated by EA, the HPA may be the main pathway
responsible for the chronic-stress allaying effects of EA.
ACKNOWLEDGEMENTS

The research and writing of this thesis is dedicated to everyone who helped along the way in spirit and presence.

Many Thanks

My husband: Nicholas Perez; Nick without you this could never have been accomplished, this is just as much mine as it is yours. You are my pillar of strength and the love of my love.

My children: Dylan Eshkevari Perez and Fenton Eshkevari Perez; you are my future, my life, my joy and my pride. In seeing my accomplishment of this goal I hope that you are inspired to reach for yours. Anything is possible with hard work and dedication, especially when you are as special as you two.

My parents: Mehrad Eshkevari and Dr. Fakhrozaman Amir Ghazanfari, I love you both so much, you have given me life, love and pride. Baba, thank you for teaching me about the philosophy of living, I am forever grateful. Maman, thank you for always being an exemplary role model in pursuing my dreams-I miss you.

My committee members: Drs Lao, and Sherman, your knowledge in your areas of expertise has made this work what it is today, I am grateful to all of you for your invaluable, scientific input.

Faculty of the department of physiology and biophysics: Drs Mulroney, Myers, Kitlinska, and Vicini, your support has been immensely helpful in achieving this goal.

Ladan Eshkevari, M.S., CRNA, L.Ac.
TABLE OF CONTENTS

ABSTRACT iii
ACKNOWLEDGEMENTS v
LIST OF TABLES xii
Chapter I 1

A. Introduction 2
   Figure 1: Models of personality linking stress to illness 4

B. Acupuncture 8
   1. Prevalence of use 8
   2. Theory of acupuncture 10
      2.1 Qi 10
      2.2 Yin and Yang: 12
         Figure 2: A pictorial depiction of yin/yang. 14
   3. Acupuncture meridians, points and TCM organs 15
      3.1 Meridians 15
         Figures 3 and 4: The Meridians. 17
         Figure 5: A stereomicroscopic image of the lymphatic vessel around the caudal
         vena cava of a rat. 19
      3.2 Acupuncture points 20
         3.2.1 Acupuncture point St 36 21
         Figure 6: Auricular acupuncture points 24
         Figure 7: Acupuncture points of the Stomach meridian 25
         Figure 8: Rat acupuncture point map 27
      3.3 Organs according to TCM 28
   4. Acupuncture treatment 31
      Figure 9: Opioid peptides and opioid receptors involved in analgesia elicited by EA
      of different frequencies 34

C. Stress 35
   1. History 35
   2. Definitions of stress, stressor and the stress response 36
   3. The paraventricular nucleus (PVN) 39
      Figure 10: Various brain areas involved in the afferent and efferent projections that
      occur with stress 41
   4. Sympathetic nervous system (SNS) 42
   5. Hypothalamus-pituitary-adrenal axis (HPA) 44
      Figure 11: Brain circuits participating in the regulation of the neuroendocrine stress
      response 46
      Figure 12: The effect of chronic stress on the brain with subsequent release of CRH,
      ACTH, NPY and CORT 47
   6. Stress related neurohormones and peptides 48
      6.1 Corticotropin releasing hormone (CRH): 48
         6.1.1 CRH1 receptor 49
         6.1.2 CRH2 receptor 50
      6.2 Adrenocorticotropic hormone (ACTH) 50
      6.3 Corticosterone (CORT) 51
6.4 Neuropeptide Y (NPY)
   6.4.1 NPY-Y1 receptor
   6.4.2 NPY-Y2 receptor
   6.4.3 NPY-Y3 and Y4 receptors
   6.4.4 NPY-Y5 and y6 receptors
6.5 Norepinephrine
   6.5.1 α1 and α2 receptors
   6.5.2 β1 and β2 receptors
   Figure 13. Pathway of synthesis for norepinephrine
7. Interaction of HPA with NPY
   7.1 NPY and catecholamines (NE)
   7.2 NPY and CRH
   7.3 NPY, ACTH and CORT
8. Cold stress as an animal model for chronic stress

D. Pharmacological Agents and the HPA
   1. Mifepristone (RU-486)
   2. Propranolol

E. Stress and acupuncture: a possible link?

Chapter II

A. Overarching hypothesis
   1.1 Aim I
   1.2 Aim II
   Figure 14: Proposed study conceptual framework

Chapter III

A. Aim I
   1. Hypothesis a/Aim I
   1.1 Experiment 1
   1.2 Experiment 2
   2. Hypothesis b/Aim I
   2.1 Experiment 3
   2.2 Experiment 4
   2.3 Experiment 5
   3. Hypothesis c/Aim I
   3.1 Experiment 6
   3.2 Experiment 7
   3.3 Experiment 8
   4. Hypothesis d/Aim I
   4.1 Experiment 9
   4.2 Experiment 10
   4.3 Experiment 11
   4.4 Experiment 12
   5. Hypothesis e/Aim I
   5.1 Experiment 13
   5.2 Experiment 14
Chapter V

A. Aim I

1. Hypothesis a/Aim I

2. Hypothesis b/Aim I

2.1 HPA Axis Hormone Levels

- Figure 18: Plasma ACTH levels on days 1 (pretreatment), 7 and 14 respectively.
- Figure 19: Serum corticosterone levels on days 1, 7 and 14 respectively.

2.2 SNS hormone levels

- Figure 20: Plasma NPY levels on days 1, 7 and 14 respectively.
- Figure 21: Analysis of plasma NE levels at day 14.

2.3 Duration of effects of post-EA treatments on chronic stress

2.3.1 HPA Hormones

- Figure 22: Plasma ACTH levels in the longitudinal study at day 14.
- Figure 23: Serum CORT levels in the longitudinal study at day 14.

2.3.2 SNS Hormones

- Figure 24: ppp NPY levels at day 14 of the longitudinal study.
- Figure 25: Plasma NE levels at day 14 of the longitudinal study.
- Figure 26: Adrenal NPY Expression.
- Figure 27: Adrenal TH Expression.

3. Hypothesis c/Aim I

3.1 HPA hormones

- Figure 28: The plasma ACTH levels of the Pre-EA cohort at days 1 and 14.
- Figure 29: Pre-EA cohorts’ serum CORT levels on days 1 and 14.

3.2 SNS Hormones

- Figure 30: Pre-EA ppp NPY levels at days 1 and 14.
- Figure 31: Pre-EA Plasma NE levels at day 14.

3.3 Expression of adrenal NPY and TH

- Figure 32: Pre-EA Adrenal NPY Expression.
- Figure 33: Pre-EA Adrenal TH Expression.

4. Hypothesis d/Aim I

4.1 HPA Hormone Levels

- Figure 34: RU-486 cohort plasma ACTH levels on days 1 and 14.
- Figure 35: The effect of RU-486 on plasma ACTH levels of animals treated with 1 hour cold stress, followed by sham-EA or EA.
- Figure 36: RU-486 cohort’s serum CORT levels on days 1 and 14.
- Figure 37: The effect of RU-486 on serum CORT levels of animals treated with 1 hour cold stress, followed by sham-EA or EA.

4.2 SNS Hormone Levels

- Figure 38: RU-486 ppp NPY levels at days 1 and 14.
- Figure 39: The effect of RU-486 on ppp NPY levels of animals treated with 1 hour cold stress, followed by sham-EA or EA.
- Figure 40: RU-486 plasma NE levels at day 14.
- Figure 41: The effect of RU-486 on plasma NE levels of animals treated with 1 hour cold stress, followed by sham-EA or EA.

4.3 Expression of adrenal NPY and TH

- Figure 42: The effect of RU-486 and EA adrenal NPY expression.
- Figure 43: The effect of RU-486 and EA adrenal TH expression.
5. Hypothesis c/Aim I

5.1 HPA Hormone Levels

Figure 44: Propranolol cohort’s plasma ACTH levels on days 1 and 14.
Figure 45: The effect of Propranolol on plasma ACTH levels of animals treated with 1 hour cold stress, followed by sham-EA or EA.
Figure 46: The propranolol cohort’s serum CORT levels at days 1 and 14.
Figure 47: The effect of Propranolol on serum CORT levels of animals treated with 1 hour cold stress, followed by sham-EA or EA.

5.2 SNS Hormone Levels

Figure 48: Propranolol cohort’s ppp NPY levels on days 1 and 14.
Figure 49: The effect of Propranolol on plasma NPY levels of animals treated with 1 hour cold stress, followed by sham-EA or EA.
Figure 50: Propranolol cohort’s day 14 plasma NE levels.
Figure 51: The effect of Propranolol on plasma NE levels of animals treated with 1 hour cold stress, followed by sham-EA or EA.

5.3 Expression of adrenal NPY and TH

Figure 52: Propranolol cohort’s adrenal NPY mRNA expression
Figure 53: Propranolol cohort’s adrenal TH mRNA expression

B. Aim II

1. Hypothesis a/Aim II
2. Hypothesis b/Aim II
3. Hypothesis c/Aim II

3.1 Central effects of EA-Analysis by qPCR

Figure 54: Brain PVN Y1 Receptor Expression in EA after Stress.
Figure 55: Brain PVN Y2 Receptor Expression in EA after Stress.
Figure 56: Brain PVN CRH Expression in pre-EA.
Figure 57: Brain PVN NPY Expression in pre-EA.
Figure 58: Brain PVN Y1R Expression in pre-EA.
Figure 59: Brain PVN Y2R Expression in pre-EA.
Figure 60: Brain PVN CRH Expression in RU-486.
Figure 61: Brain PVN NPY Expression in RU-486.
Figure 62: Brain PVN Y1R Expression in RU-486.
Figure 63: Brain PVN Y2R Expression in RU-486.
Figure 64: Brain PVN CRH Expression in the propranolol cohort.
Figure 65: Brain PVN NPY Expression in the propranolol cohort.
Figure 66: Brain PVN Y1 Receptor Expression in the propranolol cohort.
Figure 67: Brain PVN Y2 Receptor Expression in the propranolol cohort.

3.2 Immunohistochemistry (IHC) of brain sections: CRH & NPY

3.2.1 CRH
3.2.2 NPY

Figure 68: PVN CRH IHC of the EA cohort (Sagittal view).
Figure 69: PVN CRH IHC of the Pre-EA cohort.
Figure 70: PVN CRH IHC of the RU-486 cohort.
Figure 71: PVN CRH IHC of the propranolol cohort.
Figure 72: PVN NPY IHC of the EA cohort (Sagittal view).
Figure 73: PVN NPY IHC of the pre-EA cohort.
Figure 74: PVN NPY IHC of the RU-486 cohort.
Figure 75: PVN NPY IHC of the propranolol cohort.

3.3 Behavioral studies for the RU-486 cohort

3.3.1 Forced Swim Test (FST)

X
LIST OF TABLES

TABLE 1:  EXAMPLES OF YIN AND YANG IN TCM THEORY .......... 14

TABLE 2: THE FIVE “SHU” POINTS OF EACH OF THE MERIDIANS. SHU POINTS ARE SOME OF THE IMPORTANT POINTS ON THE MERIDIANS, WHERE THE QI OF THE MERIDIAN BEGINS TO POUR. ST 36 IS HIGHLIGHTED AS IT IS THE POINT UTILIZED IN THIS INVESTIGATION. ............................................................ 26

TABLE 3. THE ORGANS, ACCORDING TO TCM. NOTE THAT THEY ARE RELATED TO MANY THINGS IN NATURE, INCLUDING COLOR, EMOTION, WEATHER, ETC. ........ 30

TABLE 4: APPLIED BIOSYSTEMS TAQMAN® PRIMERS AND PROBES USED IN QPCR ANALYSIS............................ 108
LIST OF FIGURES

FIGURE 1: MODELS OF PERSONALITY LINKING STRESS TO ILLNESS ................................................................. 4

FIGURE 2: A PICTORIAL DEPICTION OF YIN/YANG. ...................... 14

FIGURES 3 AND 4: THE MERIDIANS .............................................. 17

FIGURE 5: A STEREOMICROSCOPIC IMAGE OF THE LYMPHATIC VESSEL AROUND THE CAUDAL VENA CAVA OF A RAT .............................................................. 19

FIGURE 6: AURICULAR ACUPUNCTURE POINTS ......................... 24

FIGURE 7: ACUPUNCTURE POINTS OF THE STOMACH MERIDIAN 25

FIGURE 8: RAT ACUPUNCTURE POINT MAP ............................ 27

FIGURE 9: OPIOID PEPTIDES AND OPIOID RECEPTORS INVOLVED IN ANALGESIA ELICITED BY EA OF DIFFERENT FREQUENCIES .......................................... 34

FIGURE 10: VARIOUS BRAIN AREAS INVOLVED IN THE AFFERENT AND EFFERENT PROJECTIONS THAT OCCUR WITH STRESS ............................................. 41

FIGURE 11: BRAIN CIRCUITS PARTICIPATING IN THE REGULATION OF THE NEUROENDOCRINE STRESS RESPONSE ........................................................................... 46

FIGURE 12: THE EFFECT OF CHRONIC STRESS ON THE BRAIN WITH SUBSEQUENT RELEASE OF CRH, ACTH, NPY AND CORT ................................................................. 47

FIGURE 13: PATHWAY OF SYNTHESIS FOR NOREPINEPHRINE .. 59

FIGURE 14: PROPOSED STUDY CONCEPTUAL FRAMEWORK ...... 77

FIGURE 15: OPEN FIELD TEST (OFT) ............................................. 99
FIGURE 16: THE FORCED SWIM TEST (FST) .................................................. 100

FIGURE 18: PLASMA ACTH LEVELS ON DAYS 1 (PRETREATMENT), 7 AND 14 RESPECTIVELY .......... 125

FIGURE 19: SERUM CORTICOSTERONE LEVELS ON DAYS 1, 7 AND 14 RESPECTIVELY .............................................. 126

FIGURE 20: PLASMA NPY LEVELS ON DAYS 1, 7 AND 14 RESPECTIVELY ........................................................ 128

FIGURE 21: ANALYSIS OF PLASMA NE LEVELS AT DAY 14 .... 129

FIGURE 22: PLASMA ACTH LEVELS IN THE LONGITUDINAL STUDY AT DAY 14 ......................................................... 133

FIGURE 23: SERUM CORT LEVELS IN THE LONGITUDINAL STUDY AT DAY 14 ........................................................ 134

FIGURE 24: PPP NPY LEVELS AT DAY 14 OF LONGITUDINAL STUDY .............................................................. 136

FIGURE 25: PLASMA NE LEVELS AT DAY 14 OF LONGITUDINAL STUDY ........................................................................... 137

FIGURE 26: ADRENAL NPY EXPRESSION ....................................... 139

FIGURE 27: ADRENAL TH EXPRESSION ......................................... 140

FIGURE 28: THE PLASMA ACTH LEVELS OF THE PRE-EA COHORT AT DAYS 1 AND 14 ............................................ 143

FIGURE 29: PRE-EA COHORTS’ SERUM CORT LEVELS ON DAYS 1 AND 14 .............................................................. 144

FIGURE 30: PRE-EA PPP NPY LEVELS AT DAYS 1 AND 14 ........ 146

FIGURE 31: PRE-EA PLASMA NE LEVELS AT DAY 14 .................. 147

FIGURE 32: PRE-EA ADRENAL NPY EXPRESSION ...................... 149

FIGURE 33: PRE-EA ADRENAL TH EXPRESSION ....................... 150
FIGURE 34: RU-486 COHORT PLASMA ACTH LEVELS ON DAYS 1 AND 14 ................................................................. 153

FIGURE 35: THE EFFECT OF RU-486 ON PLASMA ACTH LEVELS OF ANIMALS TREATED WITH 1 HOUR COLD STRESS, FOLLOWED BY SHAM-EA OR EA .............. 154

FIGURE 36: RU-486 COHORT'S SERUM CORT LEVELS ON DAYS 1 AND 14 .................................................................. 156

FIGURE 37: THE EFFECT OF RU-486 ON SERUM CORT LEVELS OF ANIMALS TREATED WITH 1 HOUR COLD STRESS, FOLLOWED BY SHAM-EA OR EA .................. 157

FIGURE 38: RU-486 PPP NPY LEVELS AT DAYS 1 AND 14 ......... 159

FIGURE 39: THE EFFECT OF RU-486 ON PPP NPY LEVELS OF ANIMALS TREATED WITH 1 HOUR COLD STRESS, FOLLOWED BY SHAM-EA OR EA .......................... 160

FIGURE 40: RU-486 PLASMA NE LEVELS AT DAY 14 .............. 161

FIGURE 41: THE EFFECT OF RU-486 ON PLASMA NE LEVELS OF ANIMALS TREATED WITH 1 HOUR COLD STRESS, FOLLOWED BY SHAM-EA OR EA .......................... 162

FIGURE 42: THE EFFECT OF RU-486 AND EA ADRENAL NPY EXPRESSION ................................................................ 164

FIGURE 43: THE EFFECT OF RU-486 AND EA ADRENAL TH EXPRESSION .................................................................. 165

FIGURE 44: PROPRANOLOL COHORT’S PLASMA ACTH LEVELS ON DAYS 1 AND 14 ......................................................... 168

FIGURE 45: THE EFFECT OF PROPRANOLOL ON PLASMA ACTH LEVELS OF ANIMALS TREATED WITH 1 HOUR COLD STRESS, FOLLOWED BY SHAM-EA OR EA .......... 169

FIGURE 46: THE PROPRANOLOL COHORT’S SERUM CORT LEVELS AT DAYS 1 AND 14 ...................................................... 171
FIGURE 47: THE EFFECT OF PROPRANOLOL ON SERUM CORT LEVELS OF ANIMALS TREATED WITH 1 HOUR COLD STRESS, FOLLOWED BY SHAM-EA OR EA. .............. 172

FIGURE 48: PROPRANOLOL COHORT’S PPP NPY LEVELS ON DAYS 1 AND 14............................................................. 174

FIGURE 49: THE EFFECT OF PROPRANOLOL ON PLASMA NPY LEVELS OF ANIMALS TREATED WITH 1 HOUR COLD STRESS, FOLLOWED BY SHAM-EA OR EA ............. 175

FIGURE 50: PROPRANOLOL COHORT’S DAY 14 PLASMA NE LEVELS. ................................................................. 177

FIGURE 51: THE EFFECT OF PROPRANOLOL ON PLASMA NE LEVELS OF ANIMALS TREATED WITH 1 HOUR COLD STRESS, FOLLOWED BY SHAM-EA OR EA. .......... 178

FIGURE 52: PROPRANOLOL COHORT’S ADRENAL NPY MRNA EXPRESSION.......................................................... 180

FIGURE 53: PROPRANOLOL COHORT’S ADRENAL TH MRNA EXPRESSION........................................................... 181

FIGURE 54: BRAIN PVN Y1 RECEPTOR EXPRESSION IN EA AFTER STRESS ................................................................. 185

FIGURE 55: BRAIN PVN Y2 RECEPTOR EXPRESSION IN EA AFTER STRESS ................................................................. 186

FIGURE 56: BRAIN PVN CRH EXPRESSION IN PRE-EA.............. 188

FIGURE 57: BRAIN PVN NPY EXPRESSION IN PRE-EA .............. 189

FIGURE 58: BRAIN PVN Y1R EXPRESSION IN PRE-EA.............. 190

FIGURE 59: BRAIN PVN Y2R EXPRESSION IN PRE-EA.............. 191

FIGURE 60: BRAIN PVN CRH EXPRESSION IN RU-486.............. 193

FIGURE 61: BRAIN PVN NPY EXPRESSION IN RU-486.............. 194

FIGURE 62: BRAIN PVN Y1R EXPRESSION IN RU-486.............. 195
FIGURE 63: BRAIN PVN Y2R EXPRESSION IN RU-486

FIGURE 64: BRAIN PVN CRH EXPRESSION IN THE PROPRANOLOL COHORT

FIGURE 65: BRAIN PVN NPY EXPRESSION IN THE PROPRANOLOL COHORT

FIGURE 66: BRAIN PVN Y1 RECEPTOR EXPRESSION IN THE PROPRANOLOL COHORT

FIGURE 67: BRAIN PVN Y2 RECEPTOR EXPRESSION IN THE PROPRANOLOL COHORT

FIGURE 68: PVN CRH IHC OF THE EA COHORT (SAGITTAL VIEW)

FIGURE 69: PVN CRH IHC OF THE PRE-EA COHORT

FIGURE 70: PVN CRH IHC OF THE RU-486 COHORT

FIGURE 71: PVN CRH IHC OF THE PROPRANOLOL COHORT

FIGURE 72: PVN NPY IHC OF THE EA COHORT (SAGITTAL VIEW)

FIGURE 73: PVN NPY IHC OF THE PRE-EA COHORT

FIGURE 74: PVN NPY IHC OF THE RU-486 COHORT

FIGURE 75: PVN NPY IHC OF THE PROPRANOLOL COHORT

FIGURE 76: BEHAVIORAL TESTING: FST-LATENCY TO IMMOBILITY PRE- AND POST- TREATMENT WITH RU-486

FIGURE 77: BEHAVIORAL TESTING: FST-BOUTS OF IMMOBILITY PRE- AND POST- TREATMENT WITH RU-486

FIGURE 78: BEHAVIORAL TESTING: FST-DURATION OF IMMOBILITY PRE- AND POST- TREATMENT WITH RU-486
FIGURE 79: BEHAVIORAL TESTING: OFT-SECTORS VISITED PRE- AND POST- TREATMENT WITH RU-486 .......................... 218

FIGURE 80: BEHAVIORAL TESTING: OFT-WALL LEANS PRE- AND POST- TREATMENT WITH RU-486 ............................. 219

FIGURE 81: BEHAVIORAL TESTING: FST-LATENCY TO IMMOBILITY PRE- AND POST- TREATMENT WITH PROPRANOLOL .......................................................... 223

FIGURE 82: BEHAVIORAL TESTING: FST-BOUTS OF IMMOBILITY PRE- AND POST- TREATMENT WITH PROPRANOLOL 224

FIGURE 83: BEHAVIORAL TESTING: FST-DURATION OF IMMOBILITY PRE- AND POST- TREATMENT WITH PROPRANOLOL .......................... 225

FIGURE 84: BEHAVIORAL TESTING: OFT-SECTORS VISITED PRE- AND POST- TREATMENT WITH PROPRANOLOL ...... 228

FIGURE 85: BEHAVIORAL TESTING: OFT-WALL LEANS PRE- AND POST- TREATMENT WITH PROPRANOLOL .......... 229
Chapter I

Background


A. Introduction

Stress has been widely studied and has quickly become the underpinning of many disease states. Chronic stress has been directly linked with cardiovascular disease and obesity; both of which are reaching epidemic proportions in the United States. The effects of chronic stress on the immune system can also lead to major diseases such as cancer and increased infections.

In addition, stress has been demonstrated to significantly reduce physical and mental tolerances of humans, and can induce the progression of existing illnesses or can cause latent disorders to become active. In the arena of disease prevention and health promotion as defined by Healthy People 2010, it is imperative that control and suppression of stress be a public health priority [1].

In terms of cardiovascular risk, chronic stress and the manner in which one perceives the world and their environment, in the context of personality has long been established [2].

Models for such mechanisms are varied. In the health behavior model personality traits influence health behavior, e.g., smoking, exercise, over
eating, or changes in health behavior in response to chronic stress. In the interactional stress model, personality itself influences coping in stressful situations which can then lead to maladaptive physical responses which lead to illness [2].

The transactional stress moderation model extends the latter model by including the bidirectional effect of personality on exposure to stressful situations and the availability of resources to reduce stress.

Finally in the constitutional predisposition model, genetic or other psychological factors influence both personality and the development of disease [2] (Figure 1).
Figure 1: Models of personality linking stress to illness [2].
Regardless of proposed mechanisms, there is a direct link between personality, stress and cardiovascular, immune, as well as neuroendocrine diseases [1, 3-6].

Obesity which has been strongly linked to numerous diseases itself has also been associated to chronic stress. Although there is some debate, the obese have been found to be twice as likely to suffer from anxiety, impaired social interaction, and depression which are in turn stress related [5, 6].

Given the vast number of diseases confounded by stress, it is extremely important and of great significance to public health to assess the modulation of stress pathways by a fairly cost-effective, minimally invasive treatment modality.

Tapping into a body of knowledge, Traditional Chinese Medicine (TCM) and acupuncture, that provides a different paradigm in addressing this disease-linked issue is therefore extremely important. Acupuncture may provide a cost-effective, relatively non-invasive, and non-pharmacologic treatment modality, that if found to be effective in reducing stress in the animal model, can then be evaluated in the human population. The
implication of positive results in humans would then be extremely important in addressing this major health dilemma facing the American healthcare system.

Numerous studies have been performed to examine the role of acupuncture and its mechanisms of action as an analgesic. Although the mechanisms of pain as a stressor are well understood [7-22]; to date, there have been very few reported studies in examining the role, if any, of acupuncture in treating stress specifically with regards to the classic stress pathways of the hypothalamus-pituitary-adrenal axis (HPA) and the sympathetic nervous system and neuropeptide Y (SNS/NPY) systems.

Utilizing an established animal model of chronic cold stress, in this dissertation, we test the hypothesis that acupuncture is a potentially useful therapeutic modality in stress by modulating the HPA and SNS/NPY systems. Additionally we propose that acupuncture can, by affecting these pathways, have an effect on the behavioral aspects of stress.
Although our study focuses on the animal model, the long term objective is to work towards a translational model that can address stress in humans.

In fact, the model for the study was established based on my clinical experience in both the practice of anesthesia and acupuncture. In clinical practice, it is common to utilize acupuncture as a “last resort” for treatment of stress and its symptoms such as insomnia, fatigue and change in appetite before resorting to medications. Additionally, patients utilize acupuncture preoperatively to minimize the stress of surgery. In anxiety situations which are closely related to stress and its perception, pharmacological agents are often prescribed. Benzodiazepines and the use of beta blockers are well established as therapeutic means to decrease anxiety related to performance. These agents are also employed pre- and peri-operatively as anxiolytics to assist with the control of stress and its effects, especially on the cardiovascular system.

Having noted the above, however, we do recognize that humans have multiple dimensions such as personality and cognitive factors, as mentioned above, that are unique and have to be considered when
studying stress and acupuncture in human populations versus animal models.

Nevertheless, we examined the behavioral reaction to our stressor and acupuncture as described in the methods section (Chapter IV).

**B. Acupuncture**

1. Prevalence of use

Acupuncture is the most common treatment modality in Traditional Chinese Medicine (TCM), and has dated back to over 2500 years ago [23]. Acupuncture has been utilized by the Chinese for treatment of numerous disease processes, and continues to be a popular modality throughout the world. According to the World Health Organization (WHO) acupuncture is useful as an adjunct therapy in the treatments of over 50 disorders including psychological and emotional disturbances [24]. In addition, in a 1997 National Institutes of Health (NIH) consensus report, acupuncture was recognized both as a useful stand-alone treatment as well as adjunct therapy for a variety of conditions [24, 25].

Although acupuncture has been utilized in several cultures for a very long time, it was not disseminated to the United States (US) until
1970s when President Richard Nixon traveled to China. At that time, one of the reporters who traveled with him, Mr. James Reston, became quite ill and was treated at a Chinese hospital by TCM methods such as acupuncture, as well as Western Medicine. Upon his return, he began to write about his experiences which triggered a national interest in TCM. This led to the invitation of numerous Chinese physicians to the US by the American medical community, in order to learn more about TCM and specifically Acupuncture.

Since then acupuncture use has grown in popularity in the US as a form of Complementary and Alternative Medicine (CAM). In 2006, Burke *et al.* published their findings by reviewing the National Health Interview Survey of thirty-one-thousand and forty-four [31,044] adults who completed the survey from all 50 states and the District of Columbia in 2002 [4]. The study was therefore a nationally representative cross-sectional survey. Their primary outcome measure was recent use of acupuncture, defined as use within the previous 12 months. They found that about 8.19 million Americans had reported lifetime use of acupuncture, with 2.13 million having utilized acupuncture in the previous year.
The authors, therefore, concluded that CAM use, and specifically acupuncture, continues to be a significant part of consumer health seeking behavior in the United States.

In the survey it was also clear that pain syndromes and allergies were the main impetus for use of acupuncture, however, after these two maladies, acupuncture was utilized next most often for anxiety and depression [25].

2. Theory of acupuncture

While many of the primary constructs of theories of acupuncture are not proven by the standards and methods of Western biomedical science, it is useful to understand the tenets of TCM in its therapeutic functions. It is also important to recognize that TCM, in particular acupuncture, has been scientifically proven to be useful in the therapy of several pathophysiologic states such as chronic and acute pain.

2.1 Qi

Acupuncture is the insertion of needles into specific acupoints which lie on particular meridians on the body to affect the energy of the individual or qi [pronounced “chi”]. The general theory of acupuncture is therefore
based on this premise that there are meridians that traverse the body, through which there is flow of energy or qi.

Qi is the fundamental substance constituting the universe, and all phenomena are produced by the changes and movement of qi [1]. Qi is the energy of the body, and meridians, and is gained in two forms; a. at birth from our parents, called congenital qi which is essentially limited in its quantity and quality providing us with our basic constitution; and b. once we are born, qi we can from food and the environment, referred to as acquired qi [26].

The quality of acquired qi basically depends on our lifestyle habits such as the food we consume our emotional balance, and physical exercise [23]. The concept of qi is extremely important in TCM as it is the basis for diagnosing diseases. Its quality and ability to freely flow in the body is the foundation for ones health. When imbalances or disruptions occur in its flow, one is considered to be in state of stress, pain or mild to severe disease depending on the extent of the imbalance and how many organs it has effects [1]. The first effect of an emotional or physical stressful situation in Chinese Medicine is to upset the movement and transformation of qi [5].
The main functions of qi are therefore to form blood from the acquired qi, to defend against pathogens, hold and support the organs and control all aspects of homeostasis and growth within the body.

2.2 Yin and Yang:

Yin and Yang are the two complementary, yet opposing interrelated forces which together with the concept of qi, discussed above, form the underpinning of TCM. Yin and Yang are mutually exclusive and together form a whole, they are rooted together and can transform into one another. When in balance they constitute a state of harmony and healthy being, however, when they are not in equilibrium disease ensues.

Theoretically, yin and yang would ideally be in complete balance, but this balance is not static, rather it is two opposing forces that are in a constant dynamic relationship with one another (Figure 2).

In teaching TCM, I often liken the yin and yang theory to that of the autonomic nervous system; whereby the parasympathetic and sympathetic nervous systems innervate all the organs and work together to maintain homeostasis, all the while opposing yet balancing
each other. They are contrasting forces which ensure the survival of the individual, both in restful and in active states. In TCM it is believed that all the organs have both yin and yang qualities but that the main organs are more yin in nature versus the secondary organs which are more yang (see discussion on TCM organs).

The quality of yin is similar to that of the activity of the parasympathetic system in the heart. It is the quieting, slowing down, nourishing, nighttime force; whereas yang is the opposite, representing more the active, functioning, and daylight activity [Table 1].
Figure 2: A pictorial depiction of yin/yang. Yin is represented by the dark portions of the circle, and yang illustrated by the lighter area. Note that yin always has the potential to become yang and vice versa—they are transforming, in balance, opposing and are rooted together.

<table>
<thead>
<tr>
<th>YIN</th>
<th>YANG</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIGHT</td>
<td>DAY</td>
</tr>
<tr>
<td>COLD</td>
<td>HEAT</td>
</tr>
<tr>
<td>RESTING</td>
<td>ACTIVITY</td>
</tr>
<tr>
<td>QUIET</td>
<td>NOISE</td>
</tr>
<tr>
<td>WINTER</td>
<td>SUMMER</td>
</tr>
<tr>
<td>INTROVERT</td>
<td>EXTROVERT</td>
</tr>
</tbody>
</table>

Table 1: Examples of yin and yang in TCM theory.
3. Acupuncture meridians, points and TCM organs

3.1 Meridians

There are a total of fourteen meridians traversing the whole body, each carrying the energy of the organ it is associated with. In addition, there are two extra meridians (Figures 3&4). The meridians run bilaterally, ventrally and dorsally along the torso, limbs and head and neck. They are responsible for circulation of qi through out the body [1].

It is via the meridians that the energy (qi) of the organs is brought to the more superficial layers of the body so that they can be tapped into to influence the qi of the corresponding organs. The meridians also allow the qi of the organs to communicate with each other so that the individual is a holistic sum of the energies of all his/her organs, and an imbalance in the energy of one organ can influence the energy and function of the others.

The evidence for existence of such meridians has long been sought out by scientists interested in the field of acupuncture. Indeed the Europeans, in particular, the French have contributed greatly to this body of evidence.
The most commonly used method in the 80s and 90s was the injection of radioactive tracers such as $^{99m}$Tc (Technetium-99m) as sodium pertechnetate [28, 31-33]. The pathways of the injected isotopes were then visualized and were anatomically superimposable with the meridians as described by TCM, but not on lymphatic or blood vessel routes [6].
Figures 3 and 4: The Meridians. The 14 meridians which traverse the whole body bilaterally, on the back, front, head, neck and limbs [Web reference 1,2].
Most recently in 2007, a group of Korean scientists set out to demonstrate the meridian pathways by use of magnetic nanoparticles (cobalt-ferrite magnetic nanoparticles coated with dye filled amorphous silica) in the rat model [34]. Utilizing a confocal laser scanning microscope they were then able to visualize uptake of the nanoparticles by threadlike structure close to lymphatic vessels, mimicking pathways of meridians (Figure 5).
Figure 5. A stereomicroscopic image of the lymphatic vessel around the caudal vena cava of a rat. The photograph (A) and its illustration (B) show the novel threadlike structure (solid arrow) that passes through the lymphatic valve (open arrow). The photograph was taken in vivo and in situ, and a piece of black paper was put under the lymphatic vessel to exhibit the target clearly. The scale bar is 100μm [34]. (From: H.Y. Johng et al.: Use of magnetic particles to visualize threadlike structures inside lymphatic vessels of rats. eCAM, 2006. 4(1): p. 77-82.)
3.2 Acupuncture points

Several studies have been conducted in an effort to demonstrate the existence of acupuncture points in distinguishing them from other sites on the body. There are 361 acupoints on the human body and numerous points on the human ear (Figures 6&7). Of these points, about seventy are used most often in treating various diseases according to TCM; a list of a few of the most potent and commonly utilized points in TCM for each meridian and organ is noted in Table 2.

In a recent study Kramer et al. [35] found that electrical skin resistance at six commonly used acupuncture points was either lower or higher compared to the surrounding areas of the skin. Additionally, several investigators have examined the effects of acupuncture at true acupoints versus placebo points in healthy human subjects via quantitative electroencephalography (qEEG) and autonomic responses such as heart rate variability (HRV) and blood pressure (BP) measurements [36-38].

These investigators have consistently found that acupuncture at verum acupuncture points causes significant activity in the fast EEG frequency, as well as large amplitude increases, where as placebo acupuncture
does not elicit the same responses. The noted changes in brain activity at valid acupoints translate to significant alteration in the autonomic nervous system activity of their subjects, as measured by electrocardiograms (ECG), HRV, and BP [15-17].

3.2.1 Acupuncture point St 36

St 36 is a well established point utilized quite often in Traditional Chinese Medicine for treating, amongst many other maladies, stress and anxiety in humans [1]. It is situated on the stomach meridian one finger width lateral from the anterior border of the tibia (Figure 7). In the rat, it is point # 16 and is located bilaterally on the hind leg lateral to the tibia, and just below the anterior tubercle of the tibia (Figure 8). This point is also easily accessed in the awake animal, minimizing the need for anesthetizing or immobilizing the animal, thus providing a useful model in studying stress.

In a recent study, Liu et al. found that injection of nerve growth factor into St 36 point in combination with rehabilitation training significantly improved compensation of cerebral function in children with cerebral palsy [39].
Fukazawa et al. utilized St 36 in their experiments, and found that this point had a significant impact on cholecystokinin of cerebrospinal fluid of rats [40]. Pharmacological data indicate that cholecystokinin peptides play an important role in the neurobiological mechanisms of stress- and anxiety-related behaviors.

Another recent human study has demonstrated that acupuncture at point St 36 increases cerebral blood flow auto-regulative function, cerebral hemisphere collateral circulation in patients with ischemic stroke [41].

Acupuncturing at St 36 has also been shown to modulate neuronal nitric oxide synthase expression in the brainstem to electroacupuncture Zusanli (ST36) in rats inducing long-lasting sympathoinhibition, vasodilatation and hypotension [42].

Finally, Senna-Fernandes et al. injected Wistar rats with sodium pertechnetate, while stimulating St 36. They examined the percentage of injected radio-pharmaceutical dose per gram of tissue (%ID/g) and found that the %ID/g varied significantly (p<0.05) between their control group and their acupuncture in group in many organs including the
brain; concluding that St 36 manipulation increases blood flow to various important organs including several areas of the rat brain [43].
Figure 6: Auricular acupuncture points. [Web reference 3]
Figure 7: Acupuncture points of the Stomach meridian.  
Red arrow is pointing to St36 the point used in our study, in the rat model [Web reference 4].
Table 2: The five “shu” points of each of the meridians. Shu points are some of the important points on the meridians, where the qi of the meridian begins to pour. St 36 is highlighted as it is the point utilized in this investigation.
Figure 8: Rat acupuncture point map. The red arrow is representing point ST 36 in humans corresponds to point # 16 in the rat model, and was the point consistently utilized in this study, bilaterally [Provided kindly by Dr Lao’s group at UMD]. The sham points (violet arrow) were near the tail of the animal.
With the advent of functional magnetic resonance imaging (fMRI), neuronal correlates of acupuncture stimulation in human brain have been elucidated by numerous investigators [44-48]. These scientists have clearly demonstrated that: a. acupuncture at verum points modulates the hypothalamus-limbic-paralimbic and neocortical network; and that b. sham acupuncture at non-points does not produce the same alterations in these brain areas.

It can, therefore be concluded that there is a difference in the consequence of needling at acupoints versus non-points in the central nervous system; leading to changes in integration of emotion, memory processing, autonomic, endocrine, immunological and sensorimotor functions [23]. These effects may therefore be the source of acupuncture’s therapeutic outcomes, such as its efficacy as an analgesic, and anxiolytic [24].

3.3 Organs according to TCM
The organs, according to TCM are not anatomic in their function as in Western medicine; rather, they are energetic and are all interconnected via the meridians. There are six main organs which are yin in nature (also referred to as “Zang” organs), Lungs, Heart, Pericardium, Kidney, Spleen and Liver; and six supporting, yang organs (termed “Fu” organs),
Large intestine, Small intestine, Triple burner (which divides the abdominal cavity into three areas, each deemed the burner or “jiao”), Urinary Bladder, Stomach and Gallbladder [1].

Each of the six main organs is associated with one of the fu organs such that the fu organ assists the zang organ in its function in handling the qi of the body; for example, the Kidney and Urinary bladder are associated. The organs and their relationships are best described by the five element theory within Chinese medicine. According to the five element theory of TCM, each organ is linked to a certain element in nature: Earth, Wood, Metal, Water, Fire, and this relationship further interrelates the organs with a particular emotion, season, and energy [5].

This theory is utilized quite frequently in diagnosis and treatment in TCM here in the US and is particularly popular amongst European acupuncture practitioners [Table 3].
<table>
<thead>
<tr>
<th></th>
<th>Wood</th>
<th>Fire</th>
<th>Earth</th>
<th>Metal</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yin Organ</td>
<td>Liver</td>
<td>Heart</td>
<td>Spleen</td>
<td>Lung</td>
<td>Kidney</td>
</tr>
<tr>
<td>Yang Organ</td>
<td>GB</td>
<td>SI</td>
<td>Stomach</td>
<td>LI</td>
<td>UB</td>
</tr>
<tr>
<td>Season</td>
<td>Spring</td>
<td>Summer</td>
<td>Late Sum</td>
<td>Fall</td>
<td>Winter</td>
</tr>
<tr>
<td>Weather</td>
<td>Wind</td>
<td>Heat</td>
<td>Damp</td>
<td>Dry</td>
<td>Cold</td>
</tr>
<tr>
<td>Opens</td>
<td>Eyes</td>
<td>Tongue</td>
<td>Mouth</td>
<td>Nose</td>
<td>Ears</td>
</tr>
<tr>
<td>Color</td>
<td>Green</td>
<td>Red</td>
<td>Yellow</td>
<td>White</td>
<td>Black</td>
</tr>
<tr>
<td>Emotion</td>
<td>Anger</td>
<td>Joy</td>
<td>Worry</td>
<td>Grief</td>
<td>Fear</td>
</tr>
<tr>
<td>Tastes</td>
<td>Sour</td>
<td>Bitter</td>
<td>Sweet</td>
<td>Pungent</td>
<td>Salty</td>
</tr>
<tr>
<td>Smells</td>
<td>Rancid</td>
<td>Burned</td>
<td>Sweet</td>
<td>Rank</td>
<td>Rotten</td>
</tr>
<tr>
<td>Sounds</td>
<td>Shout</td>
<td>Laugh</td>
<td>Sing</td>
<td>Cry</td>
<td>Groan</td>
</tr>
</tbody>
</table>

Table 3. The organs, according to TCM. Note that they are related to many things in nature, including color, emotion, weather, etc.
4. Acupuncture treatment

After utilizing TCM methods to diagnose an aberrant movement of qi, leading to disease; the licensed acupuncturist inserts sterile needles into specific acu-points on the meridians. By doing so, the practitioner aims to stimulate normal passage of qi, thereby resolving the symptoms and root cause of underlying pathophysiology that may be occurring.

The acupuncturist not only inserts needles into acupuncture points, but stimulates them intermittently to induce the movement of qi. The stimulation can be achieved manually by rotating the needles in a variety of directions, such as clockwise or counterclockwise, or by utilizing electricity. It is this stimulation of the points by the needle that is believed to cause the stagnant qi to move and result in relieving the patient’s symptoms.

In the recent decades the introduction of electroacupuncture (EA) has added the benefit of delivering stimulation to several acupuncture points simultaneously, making the treatment more efficient than in the past. Most current acupuncture practice as well as research is conducted utilizing EA.
The frequency of current selected for treatment varies depending on the desired outcome. Several studies have implicated the stimulation of various receptors with subsequent release of endogenous substances based on the frequency of the current applied to the acupuncture needle [49-52]. Low frequency (2-15 Hz) and high frequency (100 Hz) of EA selectively induces the release of enkephalins and dynorphins respectively in both experimental animals and humans [28-31]. Similarly, EA analgesia is thought to be mediated by mu and delta opioid receptors at 2-15 Hz and by kappa opioid receptor at 100 Hz [30,31] (Figure 9).

The duration of an acupuncture treatment has also been investigated, with a typical recommended prescription calling for 20 minutes of needling to produce the desired effect [5, 31]. This regimen is typically continued for six to eight treatments, as needed in humans.

For our experiments, the acupoints were stimulated for a period of two weeks, twenty minutes daily. The current and frequency were 2mA and 10Hz respectively and the pulse width was 0.1msec. These parameters were delivered via EA Machine Digital Electronic Acupunctoscope.
Model AWQ-104L (Lhasa Medical, Hong Kong). The output was confirmed via an oscilloscope Model 2000 (JDR Instruments, MA, USA).
Figure 9: Opioid peptides and opioid receptors involved in analgesia elicited by EA of different frequencies. (a) 2, 15 and 100 Hz. Dyn (dynorphin A); β-End (β-endorphin); Em (endomorphin); Enk (enkephalins). Stimulation at 2 Hz facilitates the release of enkephalin and at 100 Hz stimulates the release of dynorphin. The overlapping areas (purple) indicate the synergistic interaction between the two peptides [31].
C. Stress

1. History

In a technical sense, stress is a term referring to a broad range of emotions, symptoms, and environmental situations. The physiologist Walter Cannon coined the word in the early 1920s, which he borrowed from physics, meaning a measure of force per unit area within a body; therefore, a body's internal distribution of force per area that reacts to external applied loads. He also introduced the term homeostasis, and the “fight or flight” adaptive stress reaction that is now known as the stress response [53]. His contributions were instrumental in defining the roles of epinephrine/norepinephrine and the sympathetic nervous system [SNS] in the stress response [3].

In the late 1940s, Hans Selye developed the theory of stress that he called the “general adaptation syndrome” [54]. He was the first physiologist to utilize the term in describing the unspecific response of the body to an external challenge. He noted that rats exposed to varying adverse agents demonstrated the same symptoms, and that these symptoms formed a pathological triad, which included: enlargement of the adrenals, atrophy of the thymus and lymph nodes,
and gastric ulcer formation. He also determined that adenocorticotropic hormone (ACTH) and cortisol were involved in the stress response [34].

Based on Selye’s work, stress is therefore defined as: “the body’s constant concerted effort to overcome any physical or mental stressor to reestablish physiological, mental, and emotional harmony or homeostasis” [55, 56].

Since Selye’s work many have done research in the area of the stress response and have found that the Hypothalamus-Pituitary-Adrenal [HPA] axis and the medullar sympathetic nervous systems are the main neuroendocrine pathway of stress and are directly related to stress-related disorders [57-71].

2. Definitions of stress, stressor and the stress response
Stress may be defined as a “state of disharmony or threatened homeostasis” caused by a stressor which leads to both physiological and psychological responses [3]. Therefore, stress is a sensed threat to homoestasis by a stressor.
Stressors are stimuli that influence the system from within or outside, thereby initiating the “stress response” [72]. Stressors can be classified into four main categories of physical, psychological, social and those that challenge the cardiovascular and metabolic system and can be acute or chronic [3].

The stress response can be either specific to the stressor, or nonspecific, and generalized producing a stress syndrome that exceeds the organism’s ability to maintain homeostasis [3]. The organism’s response to stress is influenced to a great degree by the distinct challenge to homostasis, as well as, the organism’s perception and ability to cope with the stressor [72]. This response is generated based on the genetics of the organism, its learned behavior due to past experiences and its evaluation of the current milieu in which the stress is occurring [73]. This response generally involves the coordinated activation of the SNS and the HPA, and is integrated in the paraventricular nucleus (PVN) of the hypothalamus in the brain.

A hallmark of the stress response is the activation of the autonomic, sympathetic nervous system. Upon acute stress the brain responds by release of norepinephrine (NE), and Neuropeptide Y (NPY) in the locus
This first wave of response occurs within seconds, followed by release of corticotropin releasing hormone (CRH). Shortly thereafter, there is an increase in the secretion of ACTH and arginine vasopressin (AVP) from the pituitary and renin from the kidneys [74, 75]. This cascade of events initially serves as a protective mechanism in the “fight or flight” response. It leads to an increase in cardiovascular tone, increased blood supply to muscles and the brain, and stimulation of immune function; with a concomitant decline in sexual activity, appetite and feeding [52].

There is next, a slower response to the stressor which involves continuous glucocorticoid secretion with a subsequent drop in gonadal steroid secretion [53].

However, with chronic stress, the system goes into an overdrive, pathological mode, where by with the chronic elevation in NE and E there are severe effects on the various systems of the body, such as the cardiovascular and immune systems. Indeed, the release of NE/E and NPY have all been well documented in the human stress response model and to some extent in the rodent models as well, especially in the
3. The paraventricular nucleus (PVN)

The PVN, a triangular area in the mid-portion of the hypothalamus, sits between the third ventricle and the fornix and consists of neurons which activate the hypothalamo-pituitary-adrenocortical axis (HPA). The rat PVN contains about 20,000 neurons which have been associated with over 20 neuropeptides and neurotransmitters including corticotropin releasing hormone (CRH) [80, 81].

Afferent innervation of the PVN originates from numerous brain areas: a. the catecholaminergic axons, b. noncatecholamine peptidergic ascending fibers of the brainstem regions such as NPY, inhibin β, enkephalin c. serotonergic fibers arising from the dorsal and midbrain raphe nuclei and finally, d. intrahypothalamic projections mainly from cells that contain pro-opiomelanocortin (POMC) [82] [Figure 10].

Efferent neuronal from the PVN project to the posterior pituitary gland, medial eminence, medulla and finally to the spinal cord. They then
terminate in preganglionic neurons in the dorsal motor nucleus of the vagus, the nucleus ambiguus, and sympathetic preganglionic neurons in the intermediolateral cell column of the spinal cord [80].
Figure 10: Various brain areas involved in the afferent and efferent projections that occur with stress. The brain is an inducer and target of corticosteroids [61]. (MR and GR are mineralocorticoid and glucocorticoid receptors respectively).
4. Sympathetic nervous system (SNS)

The sympathetic nervous system has both a central and a peripheral pathway which are both activated in response to stress.

Centrally, as mentioned above, SNS neurons contain numerous important neurotransmitters and peptides including the catecholamine, NE and the neuropeptide, NPY. Catecholaminergic neurons are widespread in the brain and establish a high number of axon collaterals with thousands of synapses, which also co-express numerous peptides such as NPY as cotransmitters [72]. NPY is one of the most abundant peptides in the CNS and is not only co-localized with NE but also with ATP [76]. NE and NPY synthesizing perikarya exist in the pons and medulla, at the locus coeruleus and the arcuate nucleus, respectively. These neurons represent the efferents of the CNS that influence SNS outflow and the peripheral response of the SNS [72, 76]. Stressful stimuli accelerate both the release and turnover of NE and NPY as they are directly involved in the central processing of the stress response [72].
Once stressful stimuli reach the CNS the signal is integrated and carried to the efferent systems of HPA or SNS which occurs via the autonomic preganglionic or somatomotor neurons [72]. These preganglionic neurons may arrive directly at the thoracic spinal cord or via the brainstem and further project to the sympathetic ganglia, called the sympathoneural system, or to the adrenal gland, known as the sympathoadrenal system where they terminate [72].

In the periphery the sympathoneural system synthesizes and releases the majority of circulating NE and NPY, respectively, whereas the sympatho adrenal system provides for only 30% of NE in the plasma. The sympathoneural system is critical to the stress response and is responsible for some of the pathophysiological processes associated with stress such as hypertension, gastrointestinal diseases and carcinomas [72]. In this response, NE, and NPY are coreleased and cause a pressor response by vasoconstricting vessels via alpha 1 receptors and Y1 receptors respectively.

The sympathoadrenal system is also activated during stress, leading to increased activity of tyrosine hydroxylase in synthesizing and storing additional NE in the adrenal medulla. Elevations in plasma NE levels
are largely dependent on the sympathoneural pathway; however the synthesis of NE is increased in the adrenals, and can lead to the priming of the neural system in response to a novel stressor.

Stress can also cause release of NPY from the adrenal medulla and the cortex in the rat, although there is some evidence that the adrenal gland may not be a major source of circulating NPY in the rat [83]. For example, adrenalectomy does not affect plasma NPY levels and furthermore, although adrenal NPY increases with age, plasma NPY levels do not [83]. In fact, the adrenals are only responsible for about 20-30% of the circulating plasma NPY noted in stress [84, 85].

5. Hypothalamus-pituitary-adrenal axis (HPA)

The zona fasciculata cells of the adrenal cortex synthesize and secrete glucocorticoids in response to adrenocorticotropic hormone (ACTH) secreted by the corticotroph cells of the anterior pituitary [86]. Glucocorticoid secretion depends on ACTH levels and on adrenal medullary activity [76].

The HPA axis is controlled by a set of neurons in the medial parvoacellular division of the PVN [62]. These neurons synthesize and
secrete corticotropin releasing hormone (CRH), as well as arginine vasopressin (AVP) and oxytocin which together stimulate ACTH. In most vertebrates there is a pronounced circadian rhythm in glucocorticoid secretion, with peaks corresponding to the onset of the active phase of the diurnal cycle [87]. It is imperative to the function of the organism that this intricate system remains in the appropriate balance.

Excessive glucocorticoid release can cause major catabolic peripheral effects such as hyperglycemia, skin fragility, muscle breakdown, proteolysis, weight gain, and increased plasma amino acids, leading to a negative nitrogen balance due to increased urea formation. The negative feedback inhibition of the glucocorticoid system therefore, plays an important role in the normal as well as abnormal function of the HPA axis [66, 76] (Figures 11&12).
Figure 11: Brain circuits participating in the regulation of the neuroendocrine stress response [55].
Synthesis and secretion continues despite high levels of CORT leading to vicious cycle of elevated CORT

Altered pituitary response to CORT

Dampened in chronic stress

Figure 12: The effect of chronic stress on the brain with subsequent release of CRH, ACTH, NPY and CORT [46].
During times of stress, stress-related afferent impulses cause the activation of the secretion of CRH, AVP and oxytocin and lead to abnormally high ACTH levels, leading to an increase in cortisol secretion. Chronic stress produces a state in which the HPA axis responsiveness is preserved in spite of elevations in glucocorticoids, demonstrating the inability of the negative feedback loop to operate normally [87]. This inability to initiate or appropriately terminate HPA responses is maladaptive and leads to multiple end organ damage.

Indeed, multiple human studies have demonstrated that the abnormal HPA axis function can lead to depression, anxiety disorder, hypertension, coronary artery disease, as well as development of insulin resistant diabetes mellitus.

6. Stress related neurohormones and peptides

6.1 Corticotropin releasing hormone (CRH):

CRH is a 41 amino acid peptide which was initially isolated in the ovine hypothalamus in the early eighties [88]. It is synthesized in the PVN, the amygdala and the hippocampus; and is positively regulated by acetylcholine, norepinephrine, histamine and serotonin and inhibited by gamma-amino butyric acid (GABA) [89]. In turn, CRH controls the
secretion of POMC products such as ACTH from the anterior pituitary’s corticotropes. In addition, CRH plays a role in the activation of the SNS and release of NPY, thereby generating the neuroendocrine, autonomic and behavioral stress responses. CRH neurons also project to the noradrenergic cells in the locus coeruleus, where the release and synthesis of norepinephrine takes place under the influence of CRH [76].

The actions of CRH are mediated through three G-protein coupled receptor subtypes, CRH1-3, with CRH3 being found most recently in catfish, but not mammals [88].

6.1.1 CRH1 receptor

CRH1 receptor is expressed in the CNS with the majority of expression occurring in the cortex, cerebellum, hippocampus, amygdala, olfactory bulb and the pituitary gland [88]. It is also expressed in the periphery in the ovary, testis and adrenal glands. Binding to of CRH to CRH1 receptor activates cyclic AMP signaling pathway and induces the stress response. It has been demonstrated that under conditions of stress, CRH and CRH1 expression in the brain rises rapidly, especially in the PVN region [76, 90].
6.1.2 CRH2 receptor

CRH2 receptor is expressed both in the CNS as well as in the choroid plexus cerebral arterioles and peripheral tissues. In the CNS CRH2 is expressed in the hypothalamus, amygdala, dorsal raphe and nucleus of solitary tract [88]. Activation of the CRH2 receptor can cause either anxiogenesis or anxiolysis depending on the brain region [76].

In general it is thought that there is a “dual organization” of the CRH receptors, such that CRH1 receptors play an important role in the acute HPA response to stress, whereas CRH2 receptors are believed to be involved in the recovery phase of this response [76].

6.2 Adrenocorticotropin hormone (ACTH)

ACTH is a 39 amino acid peptide, with the primary action of stimulating growth and affecting steroid production in the adrenal cortex [91]. Secretion of ACTH is stimulated by various stressors under the influence of CRH.

ACTH acts on membrane receptors in the zona fasciculata and zona reticularis of the adrenal gland to increase cAMP levels and activates
proteinkinase A (PKA) and increase in intracellular \( \text{Ca}^{++} \) which in turn lead to synthesis and release of glucorticoids such as corticosterone [91, 92]. Conversely, normal basal ACTH secretion depends mostly on the negative feedback system, whereby, a rise in corticosterone levels inhibits the release of ACTH [93, 94].

**6.3 Corticosterone (CORT)**

Corticosterone is an important mediator of stress response. It is the primary glucorticoid in rodents and has many actions that include: increasing the rate and strength of heart contractions and sensitizing blood vessels to the actions of noradrenalin. It also has many effects on metabolic function which assist the body in coping with stressful situations.

CORT is also a potent immunoregulator and anti-inflammatory agent that plays a crucial role in preventing the immune system from overreacting to injuries and damaging tissues [1].

Corticosterone binds with high affinity to specific glucocorticoid receptors (GRs) which are mostly found in the cytoplasm during times of stress. GRs possess several functional domains, including a ligand
binding domain, a DNA binding domain and two domains which are involved in transactivation of genes once binding to DNA has occurred via interactions with other proteins [95]. By binding to a protein complex which includes two subunits of heat shock protein 90 (Hsp 90) the GR is retained in the cytosol. The heat shock proteins act as chaperones, preventing migration of the unoccupied GR to the nucleus [95]. Once GR-CORT binding takes place, GR dissociates from Hsp90 transforming into an activated, highly phosphorylated form which allows nuclear localization with subsequently binding to structures in the nucleus [75].

The activated hormone-receptor complex then causes direct or indirect activation and regulation of several target genes, thus mobilizing stored energy and increasing cardiovascular tone as discussed above.

One of the important functions of this complex is also to activate the negative feedback suppression by glucocorticoids of POMC gene to suppress further release of CRH, ACTH and glucorticoids. However, during times of chronic stress, prolonged exposure to glucocorticoids such as corticosterone leads to an impaired negative feedback system with continued elevation of corticosterone from baseline. This leads to
numerous deleterious effects such as insulin-resistant diabetes, hypertension, and immunosuppression [1, 74-76].

In addition, this chronic increase in circulating CORT levels leads to impaired brain activity, memory and cognition. These changes cause the development of anxiety and depression and an inability to cope with stressors [75].

6.4 Neuropeptide Y (NPY)

NPY is a 36 amino acid primarily synthesized and released by neurons both centrally and peripherally. Centrally it is produced in the neurons of the arcuate nucleus (ARC) which project to the PVN, noradrenergic cell bodies of the medulla, and the amygdala; while peripherally it is predominantly released via sympathetic neurons and is colocalized with norepinephrine and adenine triphosphate (ATP) [96-98]. It has numerous functions including regulation of blood pressure, feeding behavior, and stress response. In fact, various stressors in animal models have provided evidence that NPY increases in brain regions such as the amygdala and the PVN in response to stress [76, 98].

There are currently six NPY receptors identified [97], Y1-Y6. Most of these receptors are G-protein-coupled receptors which are associated
with pertussis-sensitive Gi/Go proteins [76]. Activation of the receptors causes inhibition of adenylyl-cyclase; however, additional signaling responses such as activation and inhibition of cellular Ca^{+ +} and K^{+} have also been elucidated [97].

**6.4.1 NPY-Y1 receptor**

Y1 receptors are present in the brain and the periphery. In the brain, Y1 receptor was first cloned in the rat and is present in the cerebral cortex, hippocampus, amygdala and hypothalamic nuclei [98]. Centrally, Y1R have been associated with anxiolytic effects especially after exposure to chronic stress as well as feeding behavior and fat deposition [98].

Peripherally NPY is released from sympathetic nerves and the adrenal medulla in times of stress, and causes vasoconstriction and increased blood pressure via Y1 receptor activity. Y1 receptors have also been linked with vascular smooth muscle cell growth [98-101].

**6.4.2 NPY-Y2 receptor**

The Y2 receptor is the most prevalent NPY receptor subtype in the CNS and has been associated with anxiogenic effects [98]. Activation of Y2 receptor peripherally causes the presynaptic inhibition of neurotransmitter release thereby decreasing postsynaptic excitability
The Y-2 receptor is also involved in vascular smooth muscle cell proliferation, angiogenesis, fat deposition and food intake [98, 102].

### 6.4.3 NPY-Y3 and Y4 receptors

Y3 receptor has only been pharmacologically characterized in the rat CNS, colon, lungs and adrenals. It has not been cloned and no specific agonists or antagonists have been described. The Y4 receptor is associated with inhibition of pancreatic secretion, and binds with greater affinity to pancreatic polypeptide and much less so to NPY [97, 103].

### 6.4.4 NPY-Y5 and y6 receptors

The Y5 receptor mediates feeding behavior and has been closely related to the Y1 receptor in its activity on the cardiovascular system and with the Y1/Y2 receptors in their angiogenic activity [102]. Furthermore, Y5 receptor has been implicated in NPY’s ability to modulate the HPA; however this has not been corroborated by others [104].

Finally the y6 receptor’s functions remain an enigma. It has been suggested that due to its truncated genomic formation in primates that it may have become nonfunctional during evolution [97].
6.5 Norepinephrine

Norepinephrine (NE) is a prevalent catecholamine found in many brain regions, adrenals and postsynaptic sympathetic nerve fibers and functions as a neurotransmitter and a hormone involved in the acute response to stress [76]. Efferent noradrenergic neurons from the locus coeruleus and the caudal nucleus of the solitary tract in the dorsolateral medulla along with other minor tracts from the medulla innervate the PVN where they communicate with CRH neurons [76, 78, 87, 105] to activate the acute response to stress.

Norepinephrine is synthesized in the chromaffin cells of the adrenal medulla from dopamine by dopamine β-hydroxylase, which is in turn made from L-Tyrosine by tyrosine hydroxylase (Figure 13).

The pharmacologic designation of noradrenergic receptors includes α1, α2 and β1, β2 receptors.

6.5.1 α1 and α2 receptors

The α1 receptors are associated with vasoconstriction and an increase in blood pressure; whereas the α2 receptors exert presynaptic inhibitory
action on the release of norepinephrine, thereby causing anxiolysis, and a decrease in BP and systemic vascular resistance.

6.5.2 \( \beta_1 \) and \( \beta_2 \) receptors

The \( \beta_1 \) receptors predominate in the myocardium, sinoatrial node and the ventricular conduction systems; therefore, \( \beta_1 \) activation causes positive inotropism, chronotropism and an increase in cardiac stroke volume.

The beta 2 receptors are located in the smooth muscle cells of blood vessels in the periphery as well as bronchial smooth muscles. Stimulation of these receptors leads to smooth muscle relaxation leading to vaso- and bronchodilation. In the myocardium the \( \beta_2 \) receptors also cause an increase in heart rate and increase inotropy, again improving cardiac output.

Activation of the above receptors by norepinephrine causes the initial reaction in the “fight or flight” response, commencing a cascade of increased awareness, increased myocardial, bronchial and muscular function. Additionally, instigating the adrenergic system both centrally and peripherally causes modulation of the aforementioned neuropeptides, hormones and neurotransmitters such as NPY, and CRH as well as other substances such as serotonin and substance P.
Indeed, there has also been growing evidence for the role of norepinephrine in the CNS in depression and anxiety disorders. Numerous classes of pharmacological agents, such as tricyclic anti-depressants and MAO inhibitors have been developed to increase norepinephrine in the brain to affect symptoms of depression and other mood disorders.
Figure 13. Pathway of synthesis for norepinephrine. [Web reference 5], tyrosine hydroxylase (red arrow) mRNA levels are examined in the adrenals in our study.
7. Interaction of HPA with NPY

In response to stress, there is an increase in plasma circulating levels of catecholamines and co-stored neuropeptides, such as NPY [106]. In fact, NPY is co-released with norepinephrine from sympathetic nerves and the adrenals. Concomitantly there is an increase in CRH, ACTH and corticosteroids from the HPA [84, 107]. Since both systems are anatomically and functionally unified, during stress they interact both in the periphery and in the CNS [107].

It has been postulated that these effects are modulated to a great extent via the Y-5 receptor. Indeed, studies utilizing Y5 specific agonists and antagonists such as NPY^{19-23} have found Y5 to be the main receptor involved [104].

7.1 NPY and catecholamines (NE)

Several studies have demonstrated that during stress, there is an increase in the expression of several genes which encode neurotransmitter biosynthesis such as NE, as well as neuropeptides such as NPY in the rat sympathetic ganglia and that they are interrelated [107-109]. Presynaptically, it has been demonstrated that NPY can induce inhibition of NE release via Y2 receptors as well as
activity at alpha 2 receptors of the catecholamine system [110]. The ability of NPY to interact with the catecholamine transmission reflects the possibility that NPY exerts a modulatory influence the catecholamine system at the presynaptic level, and is validated by the NPY-induced reduction in activity [110].

Peripherally, NE and NPY are co-localized in the sympathoneural system and are coreleased in response to stress, causing an increase in blood pressure and vascular remodeling [111]. Furthermore, NPY has been shown to increase catecholamine release from the rat adrenal gland via the Y1 receptor; as well as play a role in regulation of the enzymes responsible for their synthesis [85].

In addition, it has been further elucidated by Cavadas et al. in an in vitro study utilizing NPY agonists and receptor antagonists, that NPY synthesized in human adrenal chromaffin cells was able to stimulate the release of catecholamines [108]. In a subsequent study using Y1 knockout mice the same investigators were able to further reveal that this action is mainly attributed to the Y1 receptor [112]. NPY and NE have therefore been found to crosstalk in the CNS, peripheral

61
sympathetic neurons as well as the adrenal gland, with NPY playing a modulating role in NE release.

7.2 NPY and CRH

NPY neuronal input to the PVN arises from the arcuate nucleus of the hypothalamus and brain stem areas such as locus coeruleus and the medulla where NPY is colocalized with catecholaminergic cells [113]. It has further been demonstrated, by light and electron microscopy as well as anterograde tracing, that NPY projections from the ARC provide direct input into CRH cell bodies in the PVN and synapse with CRH neurons [114].

The activity of NPY neurons in the PVN is increased in several rodent models of stress, including cold stress, which subsequently leads to an increase in hypothalamic CRH mRNA synthesis followed by an increase in plasma corticosterone levels [113, 115, 116]. Additionally, the reverse has also been demonstrated, in that under acute conditions, CRH is an important mediator in initiating an increase in NPY levels [117, 118]. It is therefore theorized that CRH is involved in commencing the stress response while NPY is necessary for compensation and homeostasis [118].
In chronic stress situations, both systems are enhanced, such that NPY and CRH are increased. However, they seem to antagonize each other at least in terms of emotional response to stress. Indeed, although NPY increases CRH in the PVN acutely, its chronic intracerebroventricular (icv) injection in the rat amygdala has an anxiolytic effect versus injection of CRH into the same brain areas. Again, in this regard, it appears that NPY antagonizes the effects of CRH in the emotional and behavioral responses to stress [119].

7.3 NPY, ACTH and CORT

It has been demonstrated that acute injection of NPY into the PVN produces increases in both ACTH and corticosterone in conscious as well as anesthetized rats [85, 104, 120, 121]. Conversely, surgical interruption of the NPY pathway from the arcuate nucleus to the PVN inhibits the response of plasma ACTH to restrain, further signifying the relationship between the PVN and the ARC and their respective neurohormones and peptides [85, 122].

Chronic infusion of NPY has also led to increased plasma corticosterone levels, causing amongst other issues, an imbalance in metabolism, blood glucose levels and increased food intake [123, 124]. In fact,
numerous studies have established an important association between centrally acting NPY and circulation CORT in the pathophysiology of obesity and its related metabolic diseases [104, 124, 125]. To that end, glucorticoid receptors have been found on neurons containing NPY in the ARC of the brain [104].

NPY and CORT therefore have a positive feedback relationship in that NPY secretion increases CORT levels in the plasma, which then induce synthesis and release of NPY in the arcuate nucleus and PVN [84].

Interestingly, opposite findings has been reported in a human sleep study. In their analysis, the authors of this study found that peripheral injection of NPY to young, healthy male volunteers caused a decrease in HPA activity as evidenced by diminished circulating ACTH and CORT [126]. The investigators explained their results, citing that some animal studies have had similar outcomes with low doses of icv NPY injection, whereas most other animal studies using higher NPY doses have had contrary results. Additionally, it has been found that with central injections of NPY, there is an initial increase in ACTH and CORT, followed by a subsequent decrease approaching normal levels, with chronic infusions [127].
8. Cold stress as an animal model for chronic stress

Cold stress has been employed in numerous studies to elicit neurohormonal, and neuropeptide activity response in stress [57, 69, 73, 94, 111, 128-142].

In their study, Zukowska-Grojec et al. (1998) utilized a cold stress model whereby their rats were placed in a cage with 1cm deep ice cold water for one hour to demonstrate stress induced vasoconstriction caused by NPY [142].

Many others have examined the effects of chronic cold stress on the hypothalamic-pituitary-adrenal function of rats. The chronic cold stress model used often is exposure of the animal to chronic intermittent cold conditions. The models do vary, with most exposing the animals to any where from 1 to 6 hours of ambient cold at 4-5 degrees Celsius for 7-21 days [57, 69, 94, 133, 135].

In all of the above studies, there were significant changes in the HPA and NPY activity related to the cold stressor regardless of the methodology utilized. There was an increase in CRH, ACTH, CORT and NPY, with a clear dampening of the HPA axis negative feedback
system as demonstrated by the investigators [57, 69, 73, 94, 111, 128-141].

To that end, we have chosen a cold stress model for our examination of the effects of acupuncture in allaying the aforementioned pathways. The model we chose was that of Dr Zukowska et al., [128] such that our rats were exposed to two weeks of being placed in novel cages filled with 1cm deep crushed ice, maintained at 0-3 degrees Celsius for 60 minutes. This treatment was maintained for 14 days.

D. Pharmacological Agents and the HPA

1. Mifepristone (RU-486)

Mifepristone (RU-486) is a synthetic steroid which binds to progesterone as well as glucocorticoid receptors, acting as an antagonist. Due to its ability to bind to progesterone receptors (PRs), it has been approved for use in humans as an abortive agent, and in treatment of endometriosis, as well as PR positive tumors [143-145].

Upon binding to glucocorticoid receptors (GRs), RU-486 stabilizes the GR-heat shock protein complex, thereby reducing the number of GRs converted from an active form to ones that fails to induce transcription.
It therefore, not only prevents complete GR transformation, but alters the ability of the complex to bind to DNA [144, 145].

The effects of RU-486 vary with the dose administered in that it can antagonize PRs, GRs at low to moderate doses. However, at high doses it has estrogen-like activity as well as increases plasma ACTH and CORT purportedly due to an elevation of adrenal production of androstenedione and testosterone [146]. To that end, the dose used in this study was daily, 5mg/kg sub-cutaneous (sc) injections, dissolved in dimethyl sulfoxide (DMSO), which is within the therapeutic range for blocking GRs [146-152].

2. Propranolol

Propranolol is a non-selective β receptor antagonist developed in the early 1950s, and is the prototype β-blocking agent with which all others are compared to. It is highly lipophilic, easily crosses the blood brain barrier antagonizing the effect of numerous neurotransmitters such as norepinephrine and serotonin causing sedation, depression and a decrease in sexual arousal [153].

There is current research on the effect of propranolol as a potential treatment for post traumatic stress disorder due to its ability to inhibit the action of norepinephrine in memory consolidation [154]. Indeed animal
and human studies have demonstrated that propranolol can modulate conditioned learning as well as traumatic memory retrieval [154-158]. Additionally propranolol is used often in management of patients to allay the cardiovascular stress of surgery.

Propranolol is metabolized by the liver to water soluble metabolites such as, 17-OH propranolol which has mild $\beta$ receptor antagonist activity with a long elimination half life [153].

Hemodynamic effects include decreased blood pressure, a drop in systemic vascular resistance, decreased heart rate and myocardial contractility. It also decreases cardiac output and rennin release [153].

Complications with its use include bradycardia, heart block, brochospasm and sedation, depression and decreased sexual drive.

Effective plasma concentrations of propranolol are between 10-100ng/ml, and are achieved via the oral or intraperitoneal (ip) routes in rodents [157, 158]. To that end, the dosage used in our study was 10mg/kg dissolved in saline and injected ip once a day.


**E. Stress and acupuncture: a possible link?**

There are currently limited studies on the possible modulation of the HPA axis/SNS system via acupuncture in chronic stress, however several investigators have examined its role in acute stress/pain models. Han et al. conducted a study to investigate the inhibitory effects of electroacupuncture (EA) on the stress response evoked by tooth-pulp stimulation in the rat model [159]. They observed that in the EA group the plasma levels of norepinephrine, epinephrine, and dopamine were significantly decreased \( p<0.05 \) compared to their control group. They were also able to demonstrate that levels of plasma ACTH, and corticosterone were significantly lower \( p<0.01 \) in their experimental group as compared to their non EA group [159].

Yang et al. examined the effect of EA on response to immobilization stress, specifically looking at norepinephrine, epinephrine levels as well as heart rate and blood pressure [160]. They found that after EA their experimental group’s heart rate, BP, epinephrine and norepinephrine levels were reduced significantly [160].

Iwa et al. were able to exhibit that EA improved restraint stress-induced delay of gastric emptying via central glutaminergic and vagal pathways in conscious rats, setting up an example of a stress rat model that
responded to EA [161]. Several other investigators have also been able to validate the effectiveness of EA in attenuating various end effects of induced stress in the rat model [50, 52, 160, 162-169]. Tian et al. found that EA significantly \( p<0.05 \) attenuated stress-induced defecation in rats with chronic visceral hypersensitivity and postulated that the modulation was occurring via the serotonergic pathway [170].

Several human studies have also been conducted to examine the effects of acupuncture on stress. Middlekauff et al. conducted a pilot study with advanced heart failure patients to determine if EA would inhibit sympathetic activation [171]. They found that sympathetic activation during mental stress is “virtually eliminated after acupuncture” at distinct acupuncture points. Indeed given the small number of participants \( n=15 \) this study would have to be reinvestigated utilizing a larger population [171].

In another study, Ogata et al. investigated whether EA would inhibit palmo-plantar sweating caused by mental stress in their sample of 23 participants [172]. They concluded that at lower frequency of 5Hz compared with 100Hz there was a significant \( p<0.01 \) decrease in palmo-plantar sweating in response to induced stress [172]. Since they
observed the various frequencies with regards to acupoints on lower versus upper extremities they concluded that perhaps the mechanism of action was evidence of the involvement of a supraspinal rhythm-generating mechanism at 5Hz [172].

Given the above findings of various investigators, and clinical experience, we hypothesize that acupuncture may affect stress in modulating the classic pathways of stress. Furthermore, by doing so, acupuncture will ameliorate the effects of the neurohormones and peptides involved in the stress response.
Chapter II

Hypotheses and Aims
A. Overarching hypothesis

It is abundantly clear that there are two distinct and interconnected pathways that are involved in the stress response, the HPA and SNS systems. Both systems have an integrated central reactivity that is followed by an increase in hormone/peptide levels peripherally, which when increased chronically, lead to a dampened negative feedback system and end organ damage [9, 82, 111, 129, 139, 173-176].

To date, there are few treatment modalities for stress as a disease. Most individuals who suffer from chronic stress do not seek treatment for it per se, rather, it is not until the stress manifests as symptoms of organ decline such as hypertension, heart disease, and depression that the individual enters the healthcare system. Even then, treatment is directed at symptom management and the organs as they become dysfunctional.

Use of psychoactive medications such as antidepressants, anxiolytics, beta-blockers and sleeping aids are among the more popular treatments
along with psychotherapy. However, the aforementioned medications all have deleterious side effects; additionally they can become habit forming and are thus undesirable.

Acupuncture has been demonstrated to alleviate chronic and acute pain and is used often in treating depression and anxiety in TCM, as previously discussed. It is clear that the pain, depression, anxiety and stress pathways share some commonality in terms of both central and peripheral modulation. It is with this in mind that we hypothesize that acupuncture, by affecting the classic pathways of stress, would reduce stress effects both centrally and peripherally.

Since there are limited studies on the consequences of acupuncture treatments on stress, we aim to elucidate its effects initially supertentorially. It is our rationale that the integration of the excitatory stress response signals to activate the HPA axis occurs in the hypothalamus (PVN, in particular) as coordinated by the CRH neurons. However, releasing CRH as a hormone and a neurotransmitter triggers the cascade of events (described in the introduction section) both centrally and peripherally. Since in chronic stress, the negative feedback in this sequence of events is compromised we also
hypothesize that acupuncture could play a role in restoring this delicate balance peripherally (Figure 14).

1. Specific aims

1.1 Aim I

Determine if acupuncture (EA) decreases the activity of the peripheral HPA axis and SNS in the stress response and recovery in the chronic stress animal model.

We hypothesize that acupuncture inhibits stress-stimulated HPA axis and SNS activities, and improves recovery from chronic stress by:

a. Reducing circulating stress hormones--ACTH and CORT.

b. Decreasing peripheral release of NPY and/or norepinephrine from the sympathetic nerves.

c. Differentially targeting adrenal NPY, and adrenal tyrosine hydroxylase. The adrenal gland is examined as it is the main peripheral organ involved in integration of the stress response.

1.2 Aim II

Determine if acupuncture reduces the stress response centrally via reducing CRH and Neuropeptide Y in the PVN of chronically stressed rats.
We hypothesize that acupuncture reduces stress by modulating the HPA and SNS in the following manner:

a. Decreasing central NPY activity in the PVN.

b. Differentially targeting NPY Y1/Y2 receptors centrally in the PVN as these are both implicated in NPY’s modulating effects on the HPA (see introduction section).

c. Decreasing central CRH secretion.

d. Acupuncture decreases the behavioral changes seen in stress, such as depression and anxiety.
Figure 14: Proposed study conceptual framework.
Chapter III

Experimental Design
A. Aim I

Determine if acupuncture (EA) decreases the peripheral stress-induced HPA axis and SNS response and recovery in the chronic stress animal model.

1. Hypothesis a/Aim I: acupuncture reduces the stress-induced activity of the HPA axis by reducing circulating stress hormones—ACTH, CORT.

1.1 Experiment 1: To explore if acupuncture reduces the above hormones and peptides, peripherally in plasma and adrenal tissue.

1.2 Experiment 2: To investigate if acupuncture’s effects in reducing the peripheral levels of hormones and peptides are transient or long lasting, over several days.

2. Hypothesis b/Aim I: acupuncture reduces stress-induced activation of the SNS by reducing Norepinephrine (NE) and NPY peripherally.
2.1 **Experiment 3**: To measure NE in the plasma to assess the sympatho-neural activity.

2.2 **Experiment 4**: To measure NPY in the plasma to further assess sympatho-neural activity.

2.3 **Experiment 5**: To investigate NPY and NE expression in the adrenals by examining NPY and tyrosine hydroxylase mRNA levels to examine sympatho-adrenal activity.

**Experiments 1-5 protocol**

Adult Sprague Dawley rats were received all with round tip (jugular vein catheters) JVC, all catheters were checked and patent. The rats were weighed, tails were marked and they were randomly assigned to the 4 groups:

- Rats 1-7 Control \([n=7]\)
- Rats 8-20 Sham Acupuncture \([n=13]\)
- Rats 21-27 Stress \([n=7]\)
- Rats 28-40 EA Acupuncture \([n=13]\)

The animals were acclimated for four days, on day four plasma and serum samples were collected before any treatments-this was considered day 1 blood sample. The protocol was then begun in a staggered fashion to allow for blood draws to be done immediately after
the stress and acupuncture treatments as described in the general methods section. In brief, the animals received 1 hour cold stress followed by 30 minutes of room temperature re-acclimation before the 20-minute acupuncture treatments.

After 7 days of treatment, blood samples were again collected in a staggered manner for midpoint analysis.

Finally on day 14 of the experiment the protocol was run and animals were sacrificed and tissues and blood samples were collected. Four animals from the sham group (animals 17-20) and 4 animals from the EA group (rats 37-40) did not receive the acupuncture treatments for the last 4 days of the experiment to answer the research question of whether acupuncture’s effects were long lasting.

3. **Hypothesis c/Aim I:** Pretreatment with acupuncture prevents the increased activity of both the HPA axis and SNS noted in chronic stress, specifically by reducing ACTH, CORT, Norepinephrine (NE) and NPY peripherally.
3.1 **Experiment 6**: To measure ACTH and CORT in animal primed with 4 days of EA and then pretreated with EA *before* initiating the stress protocol. Determine if pretreatment with EA attenuates the stress response.

3.2 **Experiment 7**: To measure plasma NE and NPY in animal primed with 4 days of EA and then pretreated with EA *before* initiating the stress protocol. Determine if pretreatment with EA attenuates the stress response.

3.3 **Experiment 8**: To measure expression of NPY and TH mRNA in the adrenal glands. Determine if pre-EA as described above, attenuates stress induced sympathoadrenal activity, by reducing mRNA expression of NPY and TH in the adrenals.

**Experiment 6-8 protocols**

Adult Sprague Dawley rats were received all with round tip JVC which were checked and patent. The rats were weighed, tails were marked and they were randomly divided into four groups:
Rats 1-6 Control [n=6]
Rats 7-16 Sham Acupuncture [n=10]
Rats 17-22 Stress [n=6]
Rats 23-32 EA Acupuncture [n=10]

The animals were acclimated similarly for four days, on day four plasma and serum samples were collected before any treatments-this was considered day 1 blood sample. The protocol was then started in a staggered fashion to again, allow for blood draws to be done immediately after the acupuncture and stress treatments as described in the general methods section. In brief, the animals received 20 minutes of either sham or EA acupuncture for 5 days. On the fifth day and for the rest of the experiment the sham-EA and EA were followed by 1 hour cold stress as in the previous experiment. Animals were then returned to their home cages.

Finally on day 14 of the experiment the protocol was run and animals were sacrificed and tissues and blood samples were collected.

4. Hypothesis d/Aim I: acupuncture reduces the activity of the HPA generated in chronic stress by reducing corticosterone activity peripherally.
4.1 Experiment 9: To examine the influence of corticosterone, CORT was inhibited using steroid receptor antagonist RU486. After 10 days of cold stress, RU 486 was injected before each stress, sham-EA or EA acupuncture treatment for 4 additional days, until the end of the study on day 14.

4.2 Experiment 10: To measure ACTH and CORT in the RU486 treated animals described above. Determine if RU486 together with EA attenuate the stress response in reducing ACTH and CORT.

4.3 Experiment 11: To measure plasma NE and NPY in the RU486 treated animals as described above. Determine if RU486 together with EA attenuate the stress response in reducing NE and NPY.

4.4 Experiment 12: To measure expression of NPY and TH mRNA in the adrenal glands as an evaluation of the sympathoadrenal stress response. Determine if EA attenuates the stress-induced mRNA expression of NPY and TH in the adrenals.
Experiment 9-12 protocol

Adult Sprague Dawley rats were received all with round tip JVC which were checked and patent. The rats were weighed, tails were marked and they were randomly divided into five groups:

Rats 1-6 Control + Vehicle (n=6)
Rats 7-12 Control + RU 486(n=6)
Rats 13-18 Stress + RU 486(n=6)
Rats 19-25 Stress + RU 486 + Sham (n=8)
Rats 25-34 Stress + RU 486 + EA (n=8)

The animals were acclimated for four days, on day four plasma and serum samples were collected before any treatments-this was considered day 1 blood sample. The protocol was then begun in a staggered fashion to allow for blood draws to be done immediately after the stress and acupuncture treatments as described in the general methods section. In brief, the animals received 1 hour cold stress followed by 30 minutes of room temperature re-acclimation before the acupuncture treatments were started.

The protocol was continued until day 9. On the 9th experimental day, the animals were brought to a lab set up for behavioral studies (see general methods section) which consisted of forced swim as well as open field tests.
On the 10th day of the experiment RU-486 was prepared as follows: 1gm RU486 [Sigma-Aldrich] was dissolved in 20mL DMSO [dimethyl sulfoxide]. Each animal was injected with 100uL (0.1mL) totaling a dose of 5mg/animal/day SQ. The SQ vehicle injections consisted of 100μL of DMSO. During this time the animals were watched very carefully for any adverse effects from the combination of RU486 and the cold stressor. All animals survived the treatments and on day 13th of the experimental day, the animals were brought to a lab set up for behavioral studies as above and received the behavioral testing treatments.

Finally on day 14 of the experiment the protocol was run and blood samples were collected. On day 15, the animals were sacrificed and tissues were harvested.

5. **Hypothesis e/Aim I**: acupuncture reduces the stress-induced activity of the SNS in chronic stress by reducing sympathoadrenal and sympathoneural activity of NE/NPY peripherally.

5.1 **Experiment 13** To examine the influence of EA on stress induced SNS NE and NPY, which are co-localized in peripheral sympathetic
neurons, and are released upon activation of sympathetic nervous system.

This aim will either support or call into question the hypothesis that EA exerts its anxiolytic effect via the sympathetic neural pathways, which are in direct communication in the CNS.

Sympathetic neurons are under direct control of the SNS, which is activated in times of stress. Additionally in chronic stress, the SNS hormones, NPY and NE both increase HPA activity centrally, via CRH, and peripherally, via the adrenal cortex.

Therefore, to explore the role of EA on stress induced SNS activated NE and NPY, both peripherally, and centrally; we blocked the sympathetic nervous system by utilizing the beta-blocker prototype drug, propranolol. Propranolol can easily cross the blood brain barrier (BBB). Therefore, by blocking the SNS with this agent, we would also be able to evaluate EA’s role in the SNS effects on the HPA.
Propranolol is useful because although it crosses the BBB it has limited sedative effects, unlike other beta-blockers which can have significant soporific effects.

**5.2 Experiment 14:** To measure ACTH and CORT in the propranolol treated animals. SNS was inhibited using beta blocker, propranolol. After 9 days of cold stress, propranolol was injected before each stress, sham-EA or EA acupuncture treatment for 5 additional days, until the end of the study on day 14. Determine if SNS blockade by propranolol attenuates EA's ability to decrease stress induced ACTH and CORT levels.

**5.3 Experiment 15:** To measure plasma NE and NPY in the propranolol treated cohorts. Determine if in the animals treated as above, propranolol attenuates EA's ability to decrease stress induced NE and NPY release.

**5.4 Experiment 16:** To measure expression of NPY and TH mRNA in the adrenal glands for evaluation of EA on the stress induced sympathoadrenal activity. Determine if in animals treated with propranolol, as described above, propranolol attenuates EA's ability to
decrease stress induced sympathoadrenal NE and NPY expression, thereby substantiating the hypothesis that EA exerts its anxiolytic effect via the sympathoadrenal pathway.

**Experiments 13-16 protocol**

Adult Sprague Dawley rats were received all with round tip JVC catheters which were checked and patent. The rats were weighed, tails were marked and they were randomly divided into five groups:

- Rats 1-6 Control + Vehicle (n=6)
- Rats 7-12 Control + Propranolol (n=6)
- Rats 13-18 Stress + Propranolol (n=6)
- Rats 19-25 Stress + Propranolol + Sham (n=8)
- Rats 25-34 Stress + Propranolol + EA (n=8)

The animals were acclimated for four days, on day five plasma and serum samples were collected before any treatments-this was considered day 1 blood sample. The protocol was then begun in a staggered fashion to allow for blood draws to be done immediately after the stress and acupuncture treatments as described in the general methods section. In brief, the animals received 1hour cold stress followed by 30 minutes of room temperature re-acclimation before the acupuncture treatments were started.
The protocol was continued until day 9. On the 9\textsuperscript{th} experimental day, the animals were brought to a lab set up for behavioral studies (see general methods section) which consisted of forced swim as well as open field tests. The propranolol group animals were then injected with propranolol 10mg/kg IP, while the vehicle control group received normal saline IP.

Propranolol was prepared as follows: 1gram propranolol [Sigma-Aldrich] was dissolved in 250ml normal saline (NS). Each animal was injected with 1ml IP totaling a dose of 4mg/animal/day. During this time the animals were again watched very carefully for any adverse effects from the combination of the beta blocker and the cold stressor. All animals survived the treatments and on day 13\textsuperscript{th} of the experimental day, the animals were brought to a lab set up for behavioral studies as above and were subjected to the behavioral testing.

Finally on day 14 of the experiment the protocol was run and blood samples were collected. On day 15, the animals were sacrificed and tissues were harvested.
**B. Aim II**

Determine if acupuncture reduces the stress response *centrally* via reducing CRH and Neuropeptide Y in the PVN of chronically stressed rats.

1. **Hypothesis a/Aim II**: acupuncture inhibits stress-provoked HPA activity by reducing CRH and NPY expression which are co-localized in the brain PVN region. Stress induced PVN NPY activation is associated with increased CRH activity; therefore it is our hypothesis that EA exerts its anxiolytic effects by decreasing NPY and CRH concomitantly in the PVN.

1.1 **Experiment 1**: Assess NPY and CRH amounts in brain sections.

1.2 **Experiment 2**: Quantify NPY, Y1R, Y2R and CRH mRNA expression levels in the PVN.

Since CRH and NPY are colocalized and in direct communication with one another, we examined the expression and immunoreactivity of both in the PVN sections of *all* the experimental group animals. Additionally we analyzed stress induced effects on mRNA expression of Y1R and
Y2R in the PVN via qPCR quantification and subsequent activity in response to sham-EA or EA.

2. **Hypothesis b/Aim II**: Acupuncture modulates stress-induced behavioral changes such as depression and anxiety, by reducing these behavior patterns.

2.1 **Experiment 1**: We assessed animal behavior by using open field test (OFT) to measure anxiety, and the forced swim test (FST) to assess depression.

*Note*: the above behavioral studies were conducted on our RU-486 and propranolol animals to further confirm our hypotheses that EA’s ability to attenuate behavioral changes is, in fact, exerted via the HPA and SNS respectively.
Chapter IV

General Methods
A. Animals

1. Animal care

Adult male, 11-12 week old, Sprague-Dawley rats [Harlan, Virginia] weighing 290-420g were included in this study. The rats were received with 23g round tip indwelling jugular vein catheters (JVCs); and were housed one per cage in a controlled environment at a constant temperature [23°C]. Upon receipt, they were weighed and randomly assigned to one of either 4 or 5 groups depending on the experiment. They were all maintained in a 12hour light/dark cycle with free access to water and regular rat chow.

All animal experiments were approved by the Georgetown University Animal Care and Use Committee [GU-IACUC] in accordance with the standards set forth by the National Institute of Health (USA) guidelines.

1.1 Treatments

1.1.1 JVC care and use- catheter care was conducted on the day after the animals had arrived, and then again on days 1, and 7 of the experiment. The maintenance consisted of drawing back on the catheter with a 1cc sterile syringe utilizing a 23g blunt tip sterile
stainless steel needles (Small Parts, Inc.), followed by instilling 0.1ml of the Harlan recommended lock solution: 5ml 100% sterile glycerin (Fisher Scientific) with 5ml 1,000U/mL Heparin Sodium.

1.1.2 Acclimation - All animals were acclimated similarly for 4 days before the onset of interventions. Acclimation included 3 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.

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

1.1.4. 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 Suzanli (Stomach, St 36) on bilateral hind legs of the animal.

1.1.5 Electroacupuncture treatments-Once the acupuncture needles described above were inserted bilaterally they were attached to electrodes. Next, the animals were gently placed singly in a novel cage with bedding and the electrodes were threaded through a hole in the cage lid. The wires were attached to an acupuncture machine (Model AWQ-104L, purchased from UPC Medical Supplies, CA) and the points were stimulated for 20 minutes at a frequency of 10Hz with 2mA output, and a pulse width of 0.1s for 20 minutes. They did not receive food or water for the duration of the 20 minutes while they received the treatment.

All acupuncture treatments occurred between 9a-12n depending on the experiment. The treatments were given either 30 minutes before the stressor or 30 minutes after the animals had acclimated to ambient room temperature, in their home cages.

1.1.6 Cold stress-The animals randomly assigned to the stress treatment groups were placed in novel solitary cages with 1 cm deep
crushed ice. The temperature of the ice mixture was maintained at 0-3°C for the duration of the stress protocol which lasted 1 hour for two weeks. After the stressor, animals were returned to their home cage.

1.1.7 Behavioral Tests
1.1.7. a. Open Field Test (OFT)-All 5 groups of animals in two of the experiments were tested in the open field test (OFT), a commonly used measure of rodent anxiety [177-180]. (Figure 15) Rats were tested 9-10 days into the stress/acupuncture protocol and then again 3-5 days post receptor blocker administration, depending on the experiment. The test entailed allowing the animal to roam freely for 6 minutes, in a box divided with tape into “sectors” with equal dimensions. Endpoints measured in this paradigm included: sectors visited, vertical rears, walls leans and defecation boli. Only the sectors visited and wall leans were eventually counted in the results, as vertical rears and defecation rarely occurred.

1.1.7. b. Forced Swim Test (FST)- (Figure 16) In order to assess depression-like responses all above groups were also tested in the forced swim test (FST), a well established behavioral test for depression, immediately following the OFT [181-192]. The forced swim test included a 5 minute period where the animal was allowed to swim in
a bucket filled with room temperature water. The animals could not reach the bottom of the bucket and were therefore forced to swim or float. Endpoints observed and measured in this paradigm included: latency to immobility, duration of immobility, bouts of immobility and defecation boli.

1.1.8 Necropsy-All animals were treated with pentobarbital 30-50mg/kg, IP and deemed unconscious as noted by unresponsiveness to noxious stimuli such as pinching the paws before commencement of organ harvesting.
Figure 15: Open field test (OFT). A box divided into equal sectors was used to test level of anxiety and depression.
Figure 16: The forced swim test (FST). Often utilized to test for depressive and hopelessness behavior.
B. Blood and tissue sampling and processing

1. Blood

Blood samples were collected between 8am-12noon for all experiments. Samples were obtained via the JVCs using 23g blunt stainless steel needles (Small Parts Inc.) in 3ml sterile syringes. Two milliliters (2ml) of blood was collected at each of 3 time points: 1. on day 1 of the experiment which before any treatment but after the 4 day acclimation period, 2. on day 7 of treatments and 3. on the 14th day of the experiment after the protocol and before sacrificing the animals. The blood draws were staggered as were the experiments.

1.1 Plasma

Plasma was collected in 1.5ml EDTA purple top microcentrifuge tubes and immediately placed on ice. In general, 1.3 ml of blood was obtained for plasma and centrifuged at 4°C at 10,000 rpm for 2 minutes. The supernatant plasma was collected in 1.5 ml low retention microcentrifuge tubes (Fisher Scientific) in 150μl aliquots. The plasma was then stored at -80°C for later analysis of ACTH, NPY and Norepinephrine via enzyme linked immunosorbent assay (ELISA) or high performance column chromatography (HPLC) respectively.
1.2 Serum
Serum was collected in 1.5ml low retention microcentrifuge tubes (Fisher Scientific) at placed on a rack at room temperature for 15 minutes. The samples were centrifuged at room temperature for 5 minutes at 5000 rpm. The supernatant serum samples were placed in 1.5ml low retention microcentrifuge tubes in 100μl aliquots and stored at -80°C for subsequent ELISA analysis of CORT.

2. Tissues
2.1 Necropsy

2.1.1 Brain harvesting and processing
Once the animal was deemed anesthetized, it was positioned prone and two incisions were made on the head using a size 15 blade. First a horizontal incision was made at the base of the skull followed by a midline incision perpendicular to the first incision. Through the two incisions, the skull was exposed and cut using heavy scissors at the foramen magnum.

The brain was carefully exposed and cut out of the skull. It was immediately placed on dry iced and cut sagittally for the first cohort of animals, but coronally for all others at midline using a surgical blade.
One half of brain tissues were snap frozen immediately in sterile RNase, DNase free 50ml centrifuge tubes for quantitative polymerase chain reaction analysis (qPCR) and stored at -80°C. The other halves were placed in sterile 50ml centrifuge tubes containing 10% buffered formalin solution and immediately placed at 4°C overnight for Immunohistochemistry (IHC).

2.1.2 Organ harvesting - The animal was placed supine after the brain harvesting, and sprayed with RNase free solution (Ambien Co. A vertical abdominal incision was made all the way up to the sternum exposing the abdominal cavity. The adrenals, kidney and stomach tissue were quickly removed and snap frozen on dry ice. The incision was then continued past the sternum and a portion of the left ventricle was also harvested and placed on dry ice. All samples were collected in 2ml RNase, DNase free sterile centrifuge tubes and immediately stored at -80°C. The adrenals were subsequently utilized for qPCR.

The animal was then placed in red, biohazard bags and taken to the appropriate drop off area in the animal facility for cremation.
C. Quantitative real-time polymerase chain reaction (qPCR)

1. RNA Isolation

Total RNA was isolated from previously resected brain paraventricular nuclei and adrenal glands from Sprague-Dawley rats using the previously described phenol-chloroform extraction method [193].

Briefly, the tissues were homogenized in 1ml of TRI reagent (Sigma-Aldrich). Phase separation was achieved using 0.1ml 1-Bromo-3-chloropropane (Sigma-Aldrich) and centrifuging the resulting mixture at 12,000g for 15 minutes at 4°C. The clear aqueous phase was removed and placed into an RNase/DNase-free microcentrifuge tube. The RNA was precipitated from the aqueous solution by adding 0.5ml isopropanol to the tube and centrifuging at 12,000g for 10 minutes at 4°C.

The remaining RNA pellet was washed with 75% ethanol, isolated by centrifugation at 7,500g for 5 minutes, dried at room temperature, and resuspended in 50μl RNase/DNase-free water. RNA purity and concentration was quantified using a NanoDrop spectrophotometer (NanoDrop® ND-1000). The samples were loaded onto the spectrophotometer and the 260nm/280nm ratio of absorbance is
obtained. This ratio is utilized to examine the purity of DNA and RNA. A ratio of \(\sim 1.8\) is generally accepted as “pure” for DNA; whereas a ratio of \(\sim 2.0\) is generally accepted as “pure” RNA. If the ratio was appreciably lower, phenol contamination was considered and the samples were treated with NaAc (3M) for further RNA precipitation. Only samples with a ratio of 1.8 or \(>\) were utilized for PCR. Most of the brain samples in one of the experiments (N=40) were degraded due to freezer issues and despite numerous attempts these tissues’ RNA quality were deemed degraded and not utilized for analysis.

2. DNase treatment and cDNA Synthesis

Removal of contaminating genomic DNA was done using the Turbo DNA-free kit (Ambion) which consisted of adding 5\(\mu\)l of 10X TURBO DNase buffer RNA to each sample and momentarily vortexing the solution. The samples were then incubated for 30 minutes at 37\(^\circ\)C, followed by a two minute incubation period at room temperature after addition of 6\(\mu\)l of DNase inactivation agent. The samples were then centrifuged at 12,000g for 1 minute and the RNA supernatant was transferred to a novel sterile centrifuge tube.
After completing DNase treatment, RNA purity and concentration was again quantified using a Nanodrop spectrophotometer. 1μg of RNA per sample was used for cDNA synthesis. Synthesis was completed using the Bio-Rad iScript cDNA Synthesis kit (Bio-Rad). Master Mix consisted of 6μl of Reaction Mix and 1.5μl of Reverse Transcriptase Solution. This aliquot was multiplied by the number of samples for example for a cohort of 34 samples, 34 X 1.5= 51μl was utilized. The appropriate amount was placed along with RNA mixture and water solution, and was quantified. Quantification of synthesized cDNA was completed using a Nanodrop spectrophotometer (NanoDrop® ND-1000) as described above.

3. Quantitative PCR

0.5μg of the previously synthesized cDNA was used in measuring the relative expression of transcripts in the Neuropeptide Y (NPY) signaling cascade as well as other genes of interest, CRH, urocortin and tyrosine hydroxylase via quantitative real-time PCR. Primer and probe sets identifying: rat Actin, GAPDH Y1, Y2, Y5, NPY, tyrosine hydroxylase, and CRH (Applied Biosystems TaqMan Gene Expression Assays, see Table 4) were used to determine relative expression of each gene. Analysis was done using the comparative Ct (cycle threshold) method.
using the housekeeping gene Actin and GAPDH for normalization. Fold induction of each gene was calculated and graphed using Prism software (GraphPad; La Jolla, CA).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
</table>
| GAPDH  | Sense: 5’-CCT TCA TTG ACC TCA ACT AC-3’  
          Antisense: 5’-GGA AGG CCA TGC CAG TGA GC-3’   |
| Beta Actin | Sense: 5’- GTG ACG TCC GTA AA-3’  
               Anti sense: 5’- CTC AGG AGG AGC AAT GAT CTC-3’ |
| TH     | Sense: 5’- GCC ATG AGC TGT TGG GAC AT -3’  
          Anti sense: 5’- CCC CAG AGA TGC AAG TCC AAT-3’   |
| CRH    | Sense: 5’- CAA TCG AGC TGT CAA GAG AGC -3’  
          Anti sense: 5’- GGA AGA AAT CCA AGG GCT GAG -3’   |
| NPY    | Sense: 5’- CAG AGG CGC CCA GAG CAG-3’  
          Anti sense: 5’- CAG CCC CAT TCG TTT GTT ACC-3’   |
| Y1R    | Sense: 5’- CTC TTG CTT ATG GRG ATG TGA-3’  
          Anti sense: 5’- CTG GAA GTT TTT GTT CAG GAA YCC A-3’   |
| Y2R    | Sense: 5’- CCT ACT GCT CCA TCA TCT TGC-3’  
          Anti sense: 5’- GTA GTT GCT GTT CAT CCA GCC   |

*Table 4: Applied Biosystems TaqMan® primers and probes used in qPCR analysis.*
D. Enzyme linked immunosorbent assay (ELISA)

An ELISA is a rapid colorimetric immunochemical test that estimates ng/ml to pg/ml amounts of biologic materials in serum, urine, or saliva. ELISA is based on the antigen-antibody binding reaction without the use of radio labeled material. It was utilized for plasma and serum measurement of levels of ACTH, NPY, Norepinephrine and corticosterone respectively.

1. ELISA for NPY and ACTH

For the NPY and ACTH ELISA methods, kits (Cat # S-1145 and S-1185 respectively) were purchased from Bachem (San Carlos, CA) and the assays were run according to the manufacturer’s recommended protocols. This kit required an initial extraction step which consisted of equilibrating a 200mg C-18 sep-column with 1ml 100% acetonitrile, followed by 1% trifluoroacetic acid (TFA, HPLC grade) 3ml, three times.

Next, the plasma was acidified to remove interfering proteins such as albumin with an equal amount (150μl) of 1% trifluoroacetic acid (TFA, HPLC grade) and centrifuged at 4°C at 15,000rpm for 25 minutes. The
supernatant was then loaded onto the previously prepared sep-columns and washed with 3ml 1% TFA twice. The columns were then placed on 5ml glass centrifuge tubes and 3ml of a second buffer (1%TFA and 60%acetonitrile with 39% distilled water) was added. The eluant was then centrifuged with a cold vacuum centrifuge to evaporate the buffer. Finally, the collected residue was re-hydrated with equal volume (150μl) of assay buffer, and used for the subsequent steps of the ELISA.

The ELISA kits provided a 96-well plates which were coated with the antibody of interest (ACTH or NPY). Each well was filled with 50μl of standard or sample in duplicate. Next, 25μl of the peptide antibody, provided in the kit, was added to each well, followed by hour incubation. The biotinylated peptide, which competes for the antibody binding sites, was added in 25μl aliquots to each well and the plate was allowed to incubate for another hour.

The plate was then washed 5 times using 200μl aliquots of wash buffer in each well. The plate was tapped vigorously to ensure complete removal of unbound biotinylated peptide. Next, streptavidin-conjugated horseradish peroxidase (SA-HRP) was added and allowed to bind to the immobilized primary antibody/ biotinylated peptide complex for 1 hour.
The plate was washed again as described above to wash away excess SA-HRP.

A substrate, 3,3',5,5'-tetramethyl benzidine dihydrochloride (TMB) was then added to the wells to react with the bound biotinylated peptide bound to the immobilized antibody. After an elapsed time, usually 30 minutes, a blue color developed. Once the color reached its maximal intensity, the reaction was stopped using 2N hydrochloric acid (HCL), and the optical density was read on a standard ELISA plate reader set at 450nm.

*Note:* For the NPY, based on an eight point standard curve, it was determined that all samples would be diluted to 10X, whereas the ACTH ELISA was carried out without dilution based on a 10 point standard curve.

2. ELISA for corticosterone

Corticosterone ELISA kit (Cat # DSL-10-81100) was purchase from Diagnostic Systems Laboratories (Webster, TX). The kit provided a corticosterone goat antibody pre-coated 96 well plate. To each well 25μl of sample and each of 8 standards was added in duplicate. Next, 100μl of rat corticosterone enzyme conjugate and 100μl of rat
corticosterone antiserum was placed in each well, and the plate was agitated on a shaker set at 500rpm for one hour. The plate was then washed using 350\(\mu\)l of assay buffer for each well, 5 times.

A substrate, TMB was added and after an elapsed time, usually 20 minutes, a blue color developed. Once the color reached its maximal intensity, the reaction was stopped using 2N hydrochloric acid (HCL), and the optical density was read on a standard ELISA plate reader set at 450nm.

3. ELISA for Norepinephrine

Although a common method for measuring catecholamines in plasma has been the use of high performance column chromatography (HPLC); in the last few years, with the advent of improved ELISA methods, there are now readily available kits which have been used with confidence by many scientists [194-197]. Indeed, Westermann, et al. and Peaston et al. (2002, 2004) examined the use of ELISA for the measurement of catecholamines compared with HPLC and concluded that the ELISA was an acceptable, rapid, accurate and reproducible assay for catecholamine determination, both clinically and in research [198, 199] as long as one catecholamine was measured at a time.
The ELISA kit (Cat # BA E-5200) we obtained was one used by the aforementioned investigators from Rocky Mountain Diagnostics (Colorado Springs, CO). The protocol used was a two step method, with the first step involving extraction and acylation. Briefly, extraction consisted of adding 50μl of assay buffer to all wells of a 96 well extraction plate, followed by an equal volume of extraction buffer. The plate was covered with foil and incubated at room temperature (RT) for 1 hour. The plate was washed using 1ml/well of assay buffer twice. Next, 150μl of acylation buffer followed by 25μl of acylation solution was added to each well and allowed to incubate for 20 minutes at RT. The plate was washed again in the same manner as above and 100μl of HCL was added to each well. The plate was incubated for 10 minutes and 90μl of the supernatant was used for the enzymatic conversion step which involved adding the 90μl of the extracted standards, controls and samples into the 96 well microtiter plate. 25μl of enzyme solution was then added and the plate was incubated for 2 hours at 37°C.

The norepinephrine ELISA was then carried out by adding 50μl of norepinephrine antiserum into all the wells from the previous plate and was incubated overnight at 4°C. The plate was washed the next day using 300μl of wash buffer per well 4 times. 100μl of enzyme conjugate
was added next and the plate allowed to incubate at RT for 30 minutes followed by a subsequent wash as above. Substrate, 100μl of TMB, was added to all wells and allowed to incubate for 20-30 minutes until the color reached the desired intensity. The reaction was then stopped with 0.25M H$_2$SO$_4$ and the optical density was read on a standard ELISA plate reader set at 450nm.

**E. High Performance Liquid Chromatography (HPLC)**

HPLC is a chromatographic procedure where high pressure is applied through the column that fractionates the different molecules of interest based on their hydrophobic characteristics and separates them using a mixture of aqueous and organic solvents as mobile phase. With this method, epinephrine, norepinephrine, and dopamine could be measured simultaneously. HPLC combined to an electrochemical detector offers the most analytical sensitivity and specificity [199]. Amounts in the order of nano-molar ranges of catecholamines could be detected from both plasma and urine.

The HPLC detection of norepinephrine was graciously conducted by Dr Eisenhofer’s laboratory for one of our cohorts. Their method, previously described (Eisenhofer et al., 1986) utilized a reversed-phase method,
whereby stainless steel C-18 columns (Waters Assoc., Milford, MA) were loaded with samples using an automated sample processor and the liquid phase was pumped at a rate of 1ml/min. Quantification was achieved by eluting the catecholamines from the column by electrochemical detection using a triple electrode system (Environmental Sciences Assoc., Bedford, MA). The assay was then validated using injected dihydroxyphenylglycol (DHPG) standards ranging in concentration from 250-1,500pg [200].

**F. Immunohistochemistry (IHC)**

Immunohistochemical staining of formalin fixed paraffin embedded rat brains were performed for CRH and NPY. Five micron sections from formalin fixed paraffin embedded tissues were deparaffinized with xylenes and rehydrated through a graded alcohol series, starting with 100% EtOH and ending with 60% EtOH.

For antigen retrieval, slides were immersed in 10 mM citrate buffer (pH 6.0) with 0.05% Tween at 98°C for 20 minutes. After blocking with 3% hydrogen peroxidase and 10% normal goat serum, slides were exposed to primary antibodies for CRH (1:2000, Abcam ab89014) and NPY (1:8000, Sigma-Aldrich 9528) for one hour at room temperature.
Horse radish peroxidase (HRP) conjugated anti-rabbit secondary antibody (Dako Envision-Plus) was applied, and the HRP was detected using DAB chromagen (Dako). Slides were counterstained with Hematoxilyn (Fisher, Harris Modified Hematoxilyn) at a 1:17 dilution for 2 minutes at RT, blued in 1% ammonium hydroxide for 1 minute at room temperature, dehydrated, and mounted with Permount. Similarly treated consecutive brain 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. The PVN was identified with the assistance of Dr Azzam, a neuroanatomist with over 40 years of experience. The various parvocellular nuclei of the PVN: dorsal parvocellular (DP), medial dorsal parvocellular (MP), and ventromedial parvocellular (VMP) areas were analyzed for staining against our two areas of importance with regards to CRH and NPY, the MP and VMP were then semi-quantitatively analyzed via the MDS Analytical Technologies Metamorph® v7.5.5.0 (Figure 17).
Areas of interest

DP-dorsal parvocellular (NE + CRH)
MC-magnocellular (AVP)

***MP-medial dorsal parvocellular (CRH+NPY)
***VMP-ventromedial parvocellular (NE+CRH+NPY)

Figure 17: Nuclei of the PVN
G. Statistical Analysis

1. General analysis methodology

A statistical analysis of this experiment was performed using the statistical software package SAS and Graphpad Prism version 4. Four statistical methods were used for this analysis:

a. Exploratory data analysis was used to summarize the dataset (not shown).

b. One-way Analysis of Variance was used to detect significant treatment effects.

c. Tukey test or Newman-Keul’s test were used to perform multiple pairwise comparisons between treatment groups.

d. Non parametric tests – Kruskal-Wallis and Wilcoxon rank sum.

1.1 Analysis of Variance

ANOVA is used to test the mean difference of hormone levels between Day 14 and Day 1 across all treatment groups, except for NE which only had data for Day 14.

One-way ANOVA is used to test for mean differences between two or more independent and normally distributed groups that are distinguished by one factor (treatment).
1.1 Multiple comparison tests

Tukey or Newman Keul’s tests were utilized to perform pair wise comparisons between treatment groups. It is a single-step multiple comparison procedure generally used in conjunction with ANOVA to identify the groups with significantly different means.

3.1 Non parametric tests – Kruskall Wallis and Wilcoxon rank sum tests

Given the small sample size and variability in some of the PCR data, the normality and equal variance assumptions required for ANOVA could be easily checked. Therefore, we use nonparametric tests to validate the results which were found significant.

Kruskall Wallis is a non-parametric method for testing equality of population medians among groups. It is identical to a one-way analysis of variance with the data replaced by their ranks. Since it is a non-parametric method, the Kruskal–Wallis test does not assume a normal population, unlike the analogous one-way ANOVA. However, the test
does assume an identically-shaped and scaled distribution for each
group, except for any difference in medians.

Wilcoxon rank sum test is a nonparametric procedure for comparing the
medians of two independent groups.

**Note:** Whenever possible we deferred to the most stringent of the
statistical analysis tools, however, when the variability of samples was
deemed an extraneous factor, the non-parametric tests mentioned
above were employed.
Chapter V

Results
**A. Aim I**

Determine if acupuncture (EA) decreases the *peripheral* stress-induced HPA axis and SNS response and recovery in the chronic stress animal model. Furthermore, to investigate if acupuncture effects on the peripheral levels of hormones and peptides are transient or long lasting.

1. **Hypothesis a/Aim I:**

acupuncture reduces the activity of the HPA axis in response to stress:

by reducing circulating stress hormones—ACTH, CORT.

2. **Hypothesis b/Aim I:**

acupuncture reduces the activity of the SNS in response to stress: by reducing circulating stress hormones and peptides—NE, NPY.

Specifically, we examined circulating stress hormone levels related to the HPA: ACTH, CORT; and to the SNS: NPY and NE via ELISA and HPLC, respectively, to address the hypothesis that acupuncture reduces the levels of these hormones peripherally. Furthermore, mRNA expression of urocortin, NPY and tyrosine hydorxylase (TH) in the adrenals was examined.

The total number of animals in this cohort was 32.
2.1 HPA Axis Hormone Levels

All plasma ACTH levels had leveled off to significantly ($p<0.001$) lower levels from control day 1, before any treatments had begun on any of the groups, when compared to day 14 (Figure 18). Plasma ACTH levels in unstressed controls was not significantly different from stressed rats on day 1 (naïve to treatment) and day 7. However, on day 14, ACTH levels were significantly higher in all the stress animals versus the controls (Figure 18). Importantly, plasma ACTH was significantly lower in stressed EA animals compared to stress only and stressed sham EA animals, suggesting that EA does affect plasma ACTH during chronic stress.
In identifying if these differences in the levels of the precursor ACTH exert physiologic action, similar analysis was performed on serum CORT levels. It was found that unlike ACTH levels which had significantly changed from day 1 to day 14, CORT levels had not been significantly affected by the time course of the study. However, by day 7, and on day 14 CORT levels were significantly lower in the unstressed-control group when compared to the sham-EA and stress groups. Conversely, the stressed-EA animals’ levels were not significantly different from controls ($p>0.05$). Furthermore, by day 14, the EA cohort’s CORT levels were significantly lower than the stress animals’ levels. This suggests that EA has a physiologically anxiolytic effect in chronically stressed animals (Figure 19).
Plasma ACTH-Days 1, 7 and 14

Figure 18: Plasma ACTH levels on days 1 (pretreatment), 7 and 14 respectively. By day 14 all ACTH levels had dropped significantly in the animals as compared to day 1 (**p<0.001). At day 14, plasma ACTH levels in the sham-EA and Stress groups were significantly elevated than in the stressed EA animals on day 14 when compared to the stress only animals (♦ p<0.05).

Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
Serum CORT-Days 1, 7 and 14

Figure 19: Serum corticosterone levels on days 1, 7 and 14 respectively. On day 7, serum CORT levels in the control, no-stress group was significantly lower than the sham-EA and stress groups (*p<0.01), whereas the EA group’s CORT levels were similar to control animals. By day 14 the unstressed control group had significantly lower serum CORT levels than the stress and sham-EA groups (*p<0.01). The EA group levels were also significantly lower than the stress group (♦p<0.05).

Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
2.2 SNS hormone levels

The platelet poor plasma (ppp) NPY levels were not significantly altered on day one or at the 7 day time points. However, at day 14 ppp NPY levels of the control unstressed group was significantly lower than the stress only and sham-EA groups (Figure 20). Subsequent analysis revealed that plasma NPY levels were brought down to near control unstressed animal levels ($p>0.05$), again suggesting that EA has physiologic effect in chronically stressed animals. Furthermore, the EA animals’ NPY levels were significantly lower than the sham-EA.

Examination of plasma NE was accomplished by sending samples to a colleague’s lab for HPLC analysis. Since the samples were shipped out of the country, and given that days 1 and 7 levels for the other stress hormones were insignificant, only plasma NE levels from day 14 were analyzed. This analysis revealed no significant difference between the groups as depicted in Figure 21.
Figure 20: Plasma NPY levels on days 1, 7 and 14 respectively. By day 14, plasma levels of the control, unstressed groups is significantly lower than the sham-EA and stress groups (*p<0.05), but not the EA group. The EA animals had significantly lower NPY levels than the sham-EA but not the stress animals (♦p<0.05, p=0.063 respectively). Analysis is by one-way ANOVA and post-hoc Newman Keul's multiple comparison test.
Figure 21: Analysis of plasma NE levels at day 14. By day 14, there was no significant difference in the NE plasma levels of the groups as compared to the unstressed control animals ($p=0.75$) despite the higher levels observed in the stress group. Analysis is by one-way ANOVA.
2.3 Duration of effects of post-EA treatments on chronic stress

To investigate if the effects of acupuncture on the peripheral levels of hormones and peptides associated with the HPA axis and SNS are transient or long lasting, we analyzed hormone levels of 8 animals, 4 in each of the sham and acupuncture groups. These animals stopped receiving electroacupuncture treatments after the cold stress for the last 4 days of the protocol while they continued to be treated with cold stress. Their hormone levels were examined using ELISA and HPLC and were subsequently compared to the control and stress groups as in the above experiments.

**Schematic diagram of the study design**

*Measurement time points:* Day 1, Day 7, Day 14

- Day 1:
  - 4 days acclimation
  - All animals $N=22$

- Day 7:
  - 14 days unstressed controls
  - $N=7$

- Day 14:
  - 14 days cold stress controls
  - $N=7$
  - 14 days cold stress followed by **10 only** days of acupuncture:
  - Sham-EA [$N=4$] or EA [$N=4$]
  - Day 14 Sacrifice
We hypothesize that acupuncture; similar to some long acting pharmacological interventions would continue to exert its effects in lowering the peripheral stress hormone levels, at least for an indefinite time.

Since the previous data suggested that 14 days of treatment appeared to be the optimum time point for assessment of efficacy of EA in the cold stress model, for the remainder of the experiments only day1 (baseline) and day 14 time points were measured.

2.3.1 HPA Hormones
At day 14 of treatment, after stopping the EA and sham, but continuing the stressor, the ACTH levels of the control, unstressed group continued to be significantly lower than the sham-EA and stress groups (Figure 22). Although the EA animals plasma ACTH levels had increased, this increase was not significant when compared to the unstressed controls ($p>0.05$). Indeed, the EA animals continued to have significantly lower ACTH levels than the stress and sham-EA animals.

The control unstressed group’s CORT levels were significantly lower than the sham group and stress animals’ levels, ($p<0.005$) (Figure 23). There was continued significant difference ($p<0.05$) between the CORT
levels of the stress only and EA groups even once EA treatments were stopped.
Figure 22: Plasma ACTH levels in the longitudinal study at day 14 are significantly lower in the control group as compared to the sham-EA and Stress experimental groups (*p<0.01). The EA animals’ ACTH levels were also significantly lower than the stress and sham-EA animals (♦ p<0.05).
Analysis is via one-way ANOVA and Newman Keul’s multiple comparison post-hoc test.
Figure 23: Serum CORT levels in the longitudinal study at day 14. The control groups’ CORT levels after the acupuncture treatments were stopped is significantly lower than the sham EA and the stress groups (*p<0.005). The EA animals continued to have significantly lower (CORT levels than the sham animals and stress animals (♦p<0.05, respectively). Analysis is by one-way ANOVA and post-hoc Newman-Keul's multiple comparison test.
2.3.2 SNS Hormones

At day 14 of the longitudinal study, ppp NPY levels of the control unstressed animals were significantly lower than the stress only group ($p<0.05$), Figure 24. The ppp NPY levels of the animals in the three cold stress groups were not significantly different, providing evidence that the effects of EA had diminished at this time.

The NE levels of the groups as analyzed by ANOVA and post-hoc analysis via Student's $t$-test revealed no significant ($p=0.47$) differences between the groups in the longitudinal study (Figure 25).
**Figure 24: ppp NPY levels at day 14 of longitudinal study.** NPY levels of the control group were significantly lower (*p<0.05) than the stress group only at the end of the longitudinal study. The effects of EA had diminished by this time, as illustrated by a rise in this cohort’s levels at this time. Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
Figure 25: Plasma NE levels at day 14 of longitudinal study. NE levels of the study groups were not significantly different in the longitudinal study ($p=0.47$). Analysis is by one-way ANOVA.
mRNA expressions of NPY and TH:

The adrenals were examined via qPCR. The adrenal NPY and TH expressions were analyzed as fold induction in comparison to beta-actin as the house keeping gene. The adrenal NPY mRNA expression did not vary significantly amongst the groups (Figure 26).

The TH mRNA expression in the adrenals of the control animals was significantly lower than the stress animals. EA had similar effect on TH expression when compared to the stress animals (Figure 27).
Figure 26: Adrenal NPY Expression. The adrenal NPY expression analyzed as fold induction against beta-actin was not statistically significant when comparing control animals the other groups. Analysis is by one-way ANOVA, also checked by Wilcoxon rank sum test.
**Figure 27: Adrenal TH Expression.** The adrenal TH expression analyzed as fold induction against beta-actin was statistically significant when comparing control animals to both sham and stress, but not against EA animals, furthermore, the EA cohort also had significantly lower levels than the stress animals (*p<0.05, for both comparisons). Analysis is by one-way ANOVA and post-hoc Newman Keul's multiple comparison test. Also checked by Wilcoxon rank sum test.
3. Hypothesis c/Aim I:

Pretreatment with acupuncture prevents the increased action of the HPA axis and SNS by maintaining peripheral baseline levels of ACTH, CORT, NPY and NE.

A cohort of 32 animals was exposed to our experimental design to ascertain the effect of pre-acupuncture (pre-EA) on the circulating neurohormones involved with the aforementioned stress pathways.

**Schematic diagram of study design**

*Measurement time points:*

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 14</th>
</tr>
</thead>
</table>
| 5 days acclimation  
All animals \(N=32\) | 14 days unstressed controls \(N=6\)                           |
|                | 14 days cold stress controls \(N=6\)                          |
|                | 14 days Sham-EA \([N=10]\) or EA \([N=10]\)  
Followed by: cold stress |

We hypothesize that pre-EA would have a stress allaying effect on the levels of neurohormones, mimicking an anxiolytic action.
3.1 HPA hormones

Plasma ACTH data for day 14 of the pre-EA experimental animals was significantly lower in the unstressed control group \( (p<0.005) \) Figure 28, when compared to the sham-EA and stress groups. However, pre-EA was effective at significantly reducing plasma ACTH levels when compared to the stress only group \( (p<0.05) \).

In the pretreatment with EA groups, the control, unstressed animals had a significantly lower serum CORT level than the sham-EA and stress groups (Figure 29). The difference between the EA and Stress group’s serum CORT levels were also significant \( (p<0.001) \) with EA returning serum CORT levels to near normal.
Figure 28: The plasma ACTH levels of the Pre-EA cohort at days 1 and 14. The control group plasma ACTH levels were significantly lower than that of the stress and sham-EA groups (*p<0.005) by day 14. Additionally, there was a significant lowering of the EA group’s ACTH levels (♦p<0.05) when compared to the stress group at this time point. Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
Figure 29: Pre-EA cohorts’ serum CORT levels on days 1 and 14. At day 14, the control, unstressed animals serum CORT levels are significantly lower than the stress group and sham groups (*p<0.001). The Pre-EA group levels were significantly lower (♦p<0.001) than that of the stress group. Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
3.2 SNS Hormones

The NPY level of the control unstressed group in the Pre-EA experiment was significantly lower ($p<0.01$) on day 14 when compared to day 1, this time effect was not observed in the other groups of animals. The unstressed control animals had significantly lower ppp NPY levels than the sham-EA and stress only groups ($p<0.001$) (Figure 30). When comparing the control and pre-EA groups to the stress and sham-EA groups, it was evident that pretreatment with acupuncture had diminished NPY levels comparable to those of the control group. There was a significant decrease in plasma NPY levels of the Pre-EA group and the control group ($p<0.01$) as compared to the stress and sham EA animals (Figure 30).

The NE levels of this cohort were analyzed using high sensitivity ELISA. There were no appreciable significant differences ($p=0.4$) in the NE levels of the groups on day 14 (Figure 31).
Figure 30: Pre-EA ppp NPY levels at days 1 and 14. NPY levels of the control unstressed group was significantly lower on day 1, when compared to day 14 (\( *p<0.01 \)), this time effect was not observed in any other group. This control group also had significantly lower ppp NPY levels than the sham-EA and the stress only groups (\( *p<0.001 \)). The NPY levels of the Pre-EA group was also significantly lower than the sham-EA and stress groups (\( ♦ p<0.01 \)). Analysis is by one-way ANOVA and post-hoc Tukey's multiple comparison test.
Figure 31: Pre-EA Plasma NE levels at day 14. The NE levels of the groups were not significantly altered by Pre-EA ($p=0.4$). Analysis is by one-way ANOVA.
3.3 Expression of adrenal NPY and TH

The adrenal tissues of the animals were analyzed for both NPY and TH. The NPY expression of the control group was significantly lower than the stress and sham-EA groups, as analyzed by comparison of fold induction to the house keeping gene beta-actin (Figure 32). The Pre-EA group’s NPY expression was similar to that of the control group, and much lower than the stress and sham-EA groups’, this difference was statistically significant.

The adrenal TH expression of the control group, when compared to the other groups was not statistically significant, although the stress alone and the sham-EA group had higher fold induction than the EA and control groups (Figure 33).
Figure 32: Pre-EA Adrenal NPY Expression. The adrenal NPY expression analyzed as fold induction against Beta-actin was statistically significant when comparing control animals to both sham and stress, as well as against Pre-EA animals (*♦ p<0.05 for all comparisons). Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
Figure 33: Pre-EA Adrenal TH Expression. The adrenal TH expression analyzed as fold induction against beta-actin was not statistically significant when comparing control animals to the other groups. Analysis is by one-way ANOVA.
4. Hypothesis d/Aim I:

acupuncture affects the stress- induced HPA by interfering with increased CORT level activity. To examine the influence of corticosterone action, CORT was inhibited using steroid receptor antagonist RU486, injected before each acupuncture session. A group of 34 animals was utilized in this experiment. The plasma hormone levels of CORT, ACTH, and NPY were examined on naïve treatment day (day 1) and 14 days after treatments. Additionally, urocortin and NPY mRNA expression was examined in the adrenal tissues of the animals.

**Schematic diagram of the study design**

*Measurement of behavior time points: Day 10  Day 13*

- **Measurement time points: Day 1**
  - 4 days acclimation
  - All animals N=34

- **Day 14**
  - 14 days unstressed controls + 4 days vehicle injection N=6
  - 14 days unstressed controls + 4 days RU injection N=6
  - 14 days cold stress controls + 4 days RU injection N=6
  - 14 days cold stress + 4 days RU injection followed by: Sham-EA or EA N=10 [for both groups]

Day 15 Sacrifice
4.1 HPA Hormone Levels

ACTH levels of the control unstressed animals in the vehicle only group was significantly lower than the stress and sham-EA groups' plasma levels ($p<0.0001$) Figure 34. ACTH levels were also significantly lowered in the EA group as compared to the stress group ($p<0.0001$). As a response to CORT receptors being blocked, the overall ACTH levels were slightly higher in this cohort than the previous two experimental groups. This prevented the effects seen in the stressed rats pre- and post-treated with EA which were not blocked.

Treatment with EA had brought the ACTH levels down to near normal with no significance ($p=0.68$) noted between the RU-control group and the EA animals.

To examine the role of RU-486 further, we analyzed the day 14 levels of plasma ACTH levels of this cohort against the day 14 levels of the animals in the first experiment since the animals had received the exact same cold stress, sham-EA and EA treatments without RU-486. Interestingly, the ACTH plasma levels of all the RU-treated animals were significantly higher than the non-RU treated cohorts at day 14, except for the EA groups' levels (Figure 35).
Figure 34: RU-486 cohort plasma ACTH levels on days 1 and 14. The control unstressed vehicle control group had significantly lower plasma ACTH levels than the sham-EA and stress groups (*p<0.001). The ACTH levels of the EA group versus the stress groups was significantly lower (♦p<0.001). Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
Figure 35: The effect of RU-486 on plasma ACTH levels of animals treated with 1 hour cold stress, followed by sham-EA or EA. After treatment with RU, the stress and sham EA animals had significantly higher plasma ACTH compared to stress and sham EA animals in the non-RU experiment. (*p<0.001 and ◊p<0.05, respectively)

Analysis is by one-way ANOVA and post-hoc Tukey's multiple comparison test.
The CORT levels of the vehicle control unstressed animals when compared to the other groups was not statistically significant \( (p=0.075) \) (Figure 36).

To examine the effect of RU-486 on serum CORT, we again analyzed the day 14 serum CORT levels of this group to that of the initial experiment. It was observed that the serum CORT levels of the stress and sham RU-treated animals were significantly lower than the serum CORT levels of their corresponding non-RU treated groups at day 14, again except the EA group’s levels (Figure 37).
Figure 36: RU-486 cohort’s serum CORT levels on days 1 and 14. The Stress group in the RU-486 experiment had the lowest CORT levels when compared to the other groups. However, the difference in the groups’ levels was not significant ($p=0.075$). Analysis is by one-way ANOVA.
Figure 37: The effect of RU-486 on serum CORT levels of animals treated with 1 hour cold stress, followed by sham-EA or EA. After treatment with RU, the stress and sham EA animals had significantly higher serum CORT levels compared to stress and sham EA animals in the non-RU experiment. (*p<0.001 and ◊ p<0.05, respectively)
Analysis is by one-way ANOVA and post-hoc Tukey's multiple comparison test.
4.2 SNS Hormone Levels

In the RU-486 cohort, the control-vehicle unstressed animals had significantly lower ppp NPY levels than the control-RU, sham-EA and EA groups’ levels, but not the EA animals (Figure 38).

To examine the effect of RU-486 on plasma NPY, we again analyzed the day 14 serum NPY levels of this group to that of the initial experiment. Plasma NPY levels of the stress and sham-EA groups of RU-treated animals were significantly lower than their non-RU treated counterparts at day 14, except the EA group’s levels (Figure 39).

The NE levels of the control groups’ animals were significantly lower than that of the stress animals but not the sham-EA or EA animals (Figure 40).

Finally, comparing the RU-treated animals’ plasma NE levels with the non-RU cohort, it was found that the stress-RU and the sham-RU animals had significantly higher plasma levels of NE at day 14, but the EA-RU animals did not (Figure 41).
Figure 38: RU-486 ppp NPY levels at days 1 and 14. At day 14 the NPY levels of the control+vehicle, unstressed animals was significantly lower than that of the control+RU, sham-EA, and EA groups (*p<0.05). Interestingly, the stress group’s NPY levels were not significantly different from the control+vehicle group.
Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
Figure 39: The effect of RU-486 on ppp NPY levels of animals treated with 1 hour cold stress, followed by sham-EA or EA. Comparing animals treated with the same stressor and sham-EA or EA protocol, it was found that the stress-RU and sham-EA-RU animals had significantly lower ppp NPY levels where as the EA-RU animals did not (*p<0.05 and ◊p<0.01, respectively)
Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
Figure 40: RU-486 plasma NE levels at day 14. The control groups had significantly lower NE levels in this cohort than the stress group (*p<0.05). Analysis is by one-way ANOVA and post-hoc Newman-Keul’s multiple comparison test.
**Figure 41:** The effect of RU-486 on plasma NE levels of animals treated with 1 hour cold stress, followed by sham-EA or EA. After treatment with RU it was found that the stress-RU and sham-EA-RU animals had significantly higher plasma NE levels compared to similarly treated animals without RU treatment (*p<0.001 and ◇p<0.05, respectively). Analysis is by one-way ANOVA and post-hoc Newman-Keul’s multiple comparison test.
4.3 Expression of adrenal NPY and TH

We further analyzed the adrenal tissue of the animals for NPY and TH expression. The NPY expression in the adrenals of the animals in the various groups was not significant, although interestingly the control+RU group had the highest fold induction (Figure 42).

The adrenal TH expression of the groups was very interesting in that the control groups and the EA+RU group had similar fold inductions, whereas the sham and stress animals had two-three times this amount and was statistically significant (Figure 43).
Figure 42: The effect of RU-486 and EA adrenal NPY expression. The Control+RU group had higher fold induction than the Stress+RU but this increase was not statistically significant. Analysis is by one-way ANOVA. Also checked by Wilcoxon rank sum test.
Figure 43: The effect of RU-486 and EA adrenal TH expression. Control animals had significantly lower TH expression than the stress and sham-EA plus RU groups, as did the EA+RU (*p<0.05). Analysis is by one-way ANOVA and post-hoc Newman-Keul’s multiple comparison test. Also analyzed by Wilcoxon rank sum test.
5. **Hypothesis e/Aim I:**

acupuncture reduces the stress-induced activity of the SNS in chronic stress by reducing the action of NE/NPY peripherally.

To examine the influence of NE and NPY which are co-localized in peripheral sympathetic neurons, and are released upon activation of sympathetic nervous system in response to stress. To block the sympathetic nervous system, we utilized the beta blocker propranolol which has limited sedative effects. Propranolol is used in clinical practice with to allay anxiety and stress, especially as it is related to sympathetic nervous system outflow and cardiovascular effects. A group of 34 animals was used to conduct this experiment.

**Schematic diagram of the study design**

*Measurement of behavior time points: Day9  Day 13*

<table>
<thead>
<tr>
<th>Measurement time points:</th>
<th>Day1</th>
<th>Day 14</th>
<th>Day 15 Sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 days acclimation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All animals N=34</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 days unstressed controls + 4 days vehicle injection N=6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 days unstressed controls + 5 days prop injection N=6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 days cold stress controls + 5 days prop injection N=6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 days cold stress + 5 days prop injection followed by: Sham-EA or EA N=10 for both groups</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Initially, the peripheral levels of HPA and SNS neurohormones were examined in this group to establish the effects of Propranolol in plasma. Next, mRNA expression levels of NPY and TH were examined in the adrenal gland.

### 5.1 HPA Hormone Levels

The plasma ACTH levels of the vehicle-control unstressed animals were significantly lower \( (p<0.0001) \) than the sham-EA and stress groups’ plasma levels (**Figure 44**). Conversely, the EA group’s plasma ACTH levels were not significantly higher than the either of the control groups, suggesting that with beta blockade, the effect of EA on chronic stress remains in tact.

To further examine the role of the non-selective SNS beta-receptor blocker, propranolol, we analyzed the day 14 levels of the ACTH of this cohort against the day 14 levels of the animals in the first experiment since the animals had received the exact same cold stress and EA treatments *without* propranolol. Interestingly, the ACTH plasma levels of all the *prop*-treated animals and the *non-prop* treated cohorts at day 14, was not significantly varied (**Figure 45**).
Figure 44: Propranolol cohort’s plasma ACTH levels on days 1 and 14. ACTH levels of the unstressed, vehicle control groups at day 14 was significantly lower than that of the sham-EA animals (*p<0.01). The unstressed control+prop group had significantly lower ACTH levels at this time point than the stress+prop and sham-EA+prop groups (**p<0.01). This difference was not seen in the EA group. Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
Figure 45: The effect of Propranolol on plasma ACTH levels of animals treated with 1 hour cold stress, followed by sham-EA or EA. Comparing animals treated with the same stressor and sham-EA or EA protocol, blockade with propranolol had no significant effect on plasma ACTH. Analysis is by one-way ANOVA.
The CORT levels of all the propranolol groups were compared using. The serum CORT level of the control-vehicle, unstressed animals was not significantly different from the other group's serum levels. However, the propranolol-control group's serum CORT levels were significantly higher than that of the stress group ($p<0.05$). This same difference was not observed in the sham-EA or EA groups in comparison to either of the unstressed control groups (Figure 46).

Additionally to examine the role of beta blockade, we analyzed the day 14 levels of the CORT of this cohort against the day 14 levels of the animals in the first experiment since the animals had received the exact same cold stress and EA treatments without propranolol. It was demonstrated that the only significant difference in comparing the two experiment’s groups of animals was that the stress group in the prop-treated cohort had much lower levels than the non-prop treated stress group (Figure 47).
Figure 46: The propranolol cohort’s serum CORT levels at days 1 and 14. The control+prop group had a significantly higher serum CORT level than the stress+prop group (*p<0.05). This difference was not observed in the sham-EA or EA groups. Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
Figure 47: The effect of Propranolol on serum CORT levels of animals treated with 1 hour cold stress, followed by sham-EA or EA. After treatment with propranolol the stress+prop animals had significant lower serum CORT levels than the stress without prop animals (*p<0.001). Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
5.2 SNS Hormone Levels

The NPY levels of the animals’ in all the groups were examined using one way ANOVA. There was no statistically significant (Figure 48).

Interestingly, the NPY levels of the three experimental groups were similar in fluctuation to that of the Control+Vehicle group in that there were no appreciable significant differences, even after 14 days of stress (Figure 48). Together, the above imply that the beta blocker had diminished any previously observed effect EA had on chronic cold stress.

Additionally, to examine the role of beta blockade we analyzed the day 14 levels of NPY of this cohort against the day 14 levels of the animals in the first experiment since the animals had received the exact same cold stress and EA treatments without propranolol. Comparing the two experiments’ animals, it was found that the stress and the sham-EA groups which had no propranolol treatments had significantly higher levels of NPY than their counterparts in the prop-treatment experiment (Figure 49).
Figure 48: Propranolol cohort’s ppp NPY levels on days 1 and 14. On day 14, all plasma NPY levels were reduced, and there were no significant statistical variances amongst the groups ($p=0.83$). Analysis is by one-way ANOVA.
PPP NPY with and without Propranolol

Figure 49: The effect of Propranolol on plasma NPY levels of animals treated with 1 hour cold stress, followed by sham-EA or EA. After treatment with propranolol the sham-EA and stress animals had significantly lowered plasma NPY levels in the stress and sham-EA animals from our previous experiment without beta-blockade (*p<0.01). Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
The NE levels of this group of animals were also deemed important as we were blocking the beta receptors directly related with the action of NE. This was accomplished by using a high sensitivity ELISA kit. It was found that there was no statistical significance in the NE levels of this cohort \( p=0.98 \) using a one way ANOVA analysis of the data (Figure 50).

Finally, to examine the role of beta blockade we analyzed the day 14 levels of NE of this cohort against the day 14 levels of the animals in the first experiment since the animals had received the exact same cold stress and EA treatments without propranolol. There was no significant differences noted between the two groups’ plasma NE levels (Figure 51).
Figure 50: Propranolol cohort’s day 14 plasma NE levels. The NE levels of the groups were similar with no statistical differences noted by ($p=0.98$). Analysis is by one-way ANOVA.
Figure 51: The effect of Propranolol on plasma NE levels of animals treated with 1 hour cold stress, followed by sham-EA or EA. Comparing animals treated with the same stressor and sham-EA or EA protocol, blockade with propranolol had no significant effect on plasma NE. Analysis is by one-way ANOVA.
5.3 Expression of adrenal NPY and TH

We then examined the mRNA expression of NPY and TH in the adrenals of this cohort as compared to the house keeping gene of reference, beta actin. The NPY mRNA expression although was lowest in the control groups, this was not statistically significant (Figure 52). The TH mRNA expression of the control+vehicle animals’ adrenals was significantly lower than the sham-EA, which had the highest fold induction of adrenal TH, perhaps identifying sham-EA as an added stressor (Figure 53).
Propranolol Adrenal NPY Expression

![Graph showing mRNA expression/beta-actin levels for different groups with 14 days of cold stress and p=0.067 vs control+veh.](image)

Figure 52: Propranolol cohort’s adrenal NPY mRNA expression. The NPY expression levels of the groups were similar with no statistical significance noted. Analysis is by one-way ANOVA; also analyzed by Kruskall Wallis and Wilcoxon rank sum tests.
Figure 53: Propranolol cohort's adrenal TH mRNA expression. Although the TH expression levels of the control+vehicle groups were lowest, it was only significantly lower than the sham-EA animals’ TH expression (*p<0.05). Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
**B. Aim II**

Determine if acupuncture reduces the induced stress response *centrally* via reducing CRH and Neuropeptide Y in the PVN of chronically stressed rats.

1. **Hypothesis a/Aim II:**

acupuncture inhibits the HPA by reducing CRH and NPY expression which are co-localized in the brain PVN region.

The CRH and NPY localization, immunoreactiviy and mRNA levels of all the above experimental animals was examined using IHC and qPCR.

2. **Hypothesis b/Aim II:**

acupuncture inhibits the HPA by reducing NPY Y1 and Y2 receptor expression which may act to control the HPA and SNS outflow in response to chronic stress.

NPY receptors Y1, Y2 mRNA expression levels were studied in the PVN.
3. Hypothesis c/Aim II

It was postulated that since acupuncture modulates the HPA and SNS centrally, that it would also affects behavioral changes seen in stress, such as depression and anxiety. To that end, the animals that received RU-486 and Propranolol were exposed to behavioral testing, given the pharmacokinetics and dynamics of these agents and their ability to cross the blood brain barrier.

3.1 Central effects of EA-Analysis by qPCR

The brain CRH and NPY mRNA expression was conducted using qPCR method for all cohorts. The mRNA expressions of the two were calculated as fold induction compared against the mRNA expression of the house keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

For the first cohort of animals there was unfortunately a freezer accident and as samples were moved the brain tissues were damaged. For this cohort, the brain was only analyzed for Y1 and Y2 receptors. The Y1 mRNA receptor expression of the control animals was significantly lower
than the sham-EA group and the stress animals \( (p<0.05) \) but not of the
EA group (Figure 54).

The Y2 receptor fold induction was not significantly varied amongst the
groups although the control and EA groups had the lowest expression of
this receptor as well (Figure 55).
Figure 54: Brain PVN Y1 Receptor Expression in EA after Stress. The control group had significantly lower Y1R expression than the sham-EA and stress groups but not the EA animals (*p<0.05 for both comparisons). Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
Figure 55: Brain PVN Y2 Receptor Expression in EA after Stress. The control group did not have significantly lower or higher Y2R expression than the other groups'. Analysis is by one-way ANOVA, also analyzed by Kruskall Wallis and Wilcoxon rank sum tests.
The brain mRNA expression of CRH, NPY, Y1R and Y2R were also examined in the pre-EA cohort. Although the brain CRH mRNA expression of both the control and pre-EA group were lower than that of the sham-EA and stress animals, this effect was only statistically significant in the control versus stress animals (Figure 56). However, the NPY mRNA expression of the control animals was significantly lower than that of the stress and sham animals but not that of the EA group, demonstrating that EA had lowered NPY expression to close to that of control animals (Figure 57). Indeed, as in the control animals, the pre-EA NPY expression was significantly lower than both the stress and sham-EA groups.

The NPY receptors Y1 expression was not significantly varied in the animals' PVNs' in this cohort, however, the Y2 expression was significantly higher in the stress group when compared to the control animals (Figures 58 & 59).
Pre-EA Study Brain PVN CRH Expression

Figure 56: Brain PVN CRH Expression in pre-EA. The control group had significantly lower brain CRH expression when compared to the stress group, but not to the sham-EA or pre-EA groups (*p<0.05). Analysis is by Kruskall Wallis and Wilcoxon rank sum tests.
Pre-EA Study Brain PVN NPY Expression

Figure 57: Brain PVN NPY Expression in pre-EA. The control group had significantly lower NPY expression in the PVN than the sham-EA and stress groups' (*p<0.01). The EA animals also had significantly lower expression of NPY than the sham-EA and stress animals' (♦p<0.05).
Analysis is by one-way ANOVA and post-hoc Newman Keul’s multiple comparison test. Also analyzed by Kruskall Wallis and Wilcoxon rank sum tests.
Pre-EA Study Brain PVN Y1 Receptor Expression

Figure 58: Brain PVN Y1R Expression in pre-EA. The control did not have significantly lower Y1R when compared to the other groups, although the stress group did have the highest fold induction. Analysis is by ANOVA. Further analysis is by Kruskall Wallis and Wilcoxon rank sum tests.
**Figure 59: Brain PVN Y2R Expression in pre-EA.** The control and pre-EA groups had significantly lower Y2R expression than the stress group but not the sham-EA (*p<0.05 for both comparisons). Analysis is by Kruskall Wallis and Wilcoxon rank sum tests.
For the RU-486 cohort, brain tissue was again analyzed via qPCR for expression of CRH, NPY, and Y1, Y2 receptors. Neither the CRH nor the NPY expression of the brain tissues' of the animals were significantly varied amongst the groups (**Figure 60 & 61**). The same was noted for the NPY Y1 and Y2 receptors in that there were no significant changes noted (**Figure 62 & 63**).
Figure 60: Brain PVN CRH Expression in RU-486. The control group did not have significantly variable CRH expression than the other groups’. Analysis is by one-way ANOVA. Further analysis is by Kruskall Wallis and Wilcoxon rank sum tests.
Figure 61: Brain PVN NPY Expression in RU-486. The control group did not have significantly variable NPY expression than the other groups'. Analysis is by one-way ANOVA also analyzed by Kruskall Wallis and Wilcoxon rank sum tests.
RU-486 Brain PVN Y1 Receptor Expression

Figure 62: Brain PVN Y1R Expression in RU-486. The control group did not have significantly variable Y1R expression than the other groups’. Analysis is by one-way ANOVA; further analysis is by Kruskall Wallis and Wilcoxon rank sum tests.
Figure 63: Brain PVN Y2R Expression in RU-486. The control group did not have significantly variable Y2R expression than the other groups'. Analysis is by one-way ANOVA; further analysis is by Kruskall Wallis and Wilcoxon rank sum tests.
For the propranolol cohort, brain tissue was also analyzed via qPCR for expression of CRH, NPY, and Y1, Y2 receptors. Neither the CRH nor the NPY expression of the brain tissues' of the animals were significantly varied amongst the groups (Figures 64 & 65). The same was noted for the NPY Y1; however the control+propranolol animals had significantly lower fold induction of Y2R than the stress group (Figure 66 & 67).
Propranolol Brain PVN CRH Expression

Figure 64: Brain PVN CRH Expression in the propranolol cohort. The control group did not have significantly variable CRH expression than the other groups'. Analysis is by one-way ANOVA; further analysis is by Kruskall Wallis and Wilcoxon rank sum tests.
Figure 65: Brain PVN NPY Expression in the propranolol cohort. The control group did not have significantly variable NPY expression than the other groups'. Analysis is by one-way ANOVA further analysis is by Kruskall Wallis and Wilcoxon rank sum tests.
Figure 66: Brain PVN Y1 Receptor Expression in the propranolol cohort.
The control group did not have significantly variable NPY Y1 expression than the other groups'.
Analysis is by one-way ANOVA; further analysis is by Kruskall Wallis and Wilcoxon rank sum tests.
Figure 67: Brain PVN Y2 Receptor Expression in the propranolol cohort. The control+prop group had significantly lower NPY Y2 expression than the stress animals (*p<0.05). Analysis is by Kruskall Wallis and Wilcoxon rank sum tests.
3.2 Immunohistochemistry (IHC) of brain sections: CRH & NPY

IHC of brain was utilized to semi-quantify the amount and localization of CRH and NPY in PVN sections. The total number of cells was counted and the green, positive staining cells were then analyzed as a percent of the all.

3.2.1 CRH-For the EA and pre-acupuncture groups the CRH immunoreactivity in the PVN was significantly lower in the control and EA groups than the stress animals (Figures 68 & 69). The same was observed in the propranolol cohort; however, in the RU-486 animals, the control animals had significantly higher CRH reactivity than the stress group (Figures 70 & 71). The EA and the sham animals also had significantly higher PVN CRH immunoreactivity (Figure 71).

3.2.2 NPY-The NPY immunoreactivity of the PVN in the EA and pre-EA cohorts was significantly lower in the control and EA animals than the stress animals (Figures 72 & 73). In the pre-EA group the expression in the EA animals was also significantly lower than that of the sham-EA group. This was also true for the RU-486
animals (Figure 74). However, interestingly, the vehicle-control animals in the propranolol cohort had significantly higher NPY immunoreactivity when compared to all the other animals including the control+propranolol animals (Figure 75).
Examining the CRH in the 4 groups, it was noted that CRH-immunoreactivity was significantly higher in the stress group when compared to the control and EA groups (*p<0.001 and ♦p<0.05 respectively). 3V-3rd ventricle. Analysis is by one-way ANOVA and post-hoc Newman Keul's multiple comparison test.

**Figure 68: PVN CRH IHC of the EA cohort (Sagittal view).**
Figure 69: PVN CRH IHC of the Pre-EA cohort. CRH-immunoreactivity was significantly higher in the stress and sham-EA groups when compared to the control and EA animals (*\(p<0.05\) respectively). 3V-3rd ventricle. Analysis is by one-way ANOVA and post-hoc Newman Keul’s multiple comparison test.
Figure 70: PVN CRH IHC of the RU-486 cohort. CRH immunoreactivity was significantly lower in the stress group as compared to the control, sham-EA and EA groups (*p<0.05). 3V-3^{rd} ventricle.
Analysis is by one-way ANOVA and post-hoc Newman-Keul’s multiple comparison test.

* p<0.05 vs stress

14 days cold stress
Figure 71: PVN CRH IHC of the propranolol cohort. CRH immunoreactivity of the control+vehicle, sham-EA and EA groups were significantly lower than the stress group (*p<0.05). 3V-3rd Ventricle. Analysis is by one-way ANOVA and post-hoc Newman-Keul's multiple comparison test.
Figure 72: PVN NPY IHC of the EA cohort (Sagittal view). NPY immunoreactivity was significantly lower in the control group as compared to the sham-Ea and stress groups (*p<0.005). The EA animals also had significantly lower NPY immunoreactivity in this brain region compared to these two cohorts (♦p<0.05). 3V-3rd ventricle. Analysis is by one-way ANOVA and post-hoc Newman-Keul’s multiple comparison test.
Figure 73: PVN NPY IHC of the pre-EA cohort. NPY immunoreactivity was significantly lower in the control group as compared to the sham-Ea and stress groups (*$p<0.001$). The EA animals also had significantly lower NPY immunoreactivity in this brain region compared to these two cohorts (♦ $p<0.05$). 3V-3rd ventricle.

Analysis is by one-way ANOVA and post-hoc Newman-Keul's multiple comparison test.
Figure 74: PVN NPY IHC of the RU-486 cohort. NPY immunoreactivity was significantly lower in the control groups’ as compared to the sham-Ea and stress groups (*p<0.001). The EA animals also had significantly lower NPY immunoreactivity in this brain region compared to these two cohorts (♦ p<0.05). 3V-3rd Ventricle.
Analysis is by one-way ANOVA and post-hoc Newman-Keul’s multiple comparison test.

* p<0.001 vs control groups
♦ p<0.05 vs EA
14 days cold stress
Figure 75: PVN NPY IHC of the propranolol cohort. NPY immunoreactivity was significantly higher in the control-vehicle groups as compared to all the groups that received propranolol: the stress, sham-EA and EA animals (*p<0.001). 3V-3rd Ventricle. Analysis is by one-way ANOVA and post-hoc Newman-Keul’s multiple comparison test.

* p<0.001 vs control+vehicle

14 days cold stress
3.3 Behavioral studies for the RU-486 cohort

There were two time points at which the behavioral studies were conducted; one was at 10 days of stress, before the RU-486 was administered (Pre-RU-486), and the other was on day 13, 3 days after the administration of the RU-486 (Post-RU-486).

3.3.1 Forced Swim Test (FST)

There were several parameters measured in the FST. The first was the time at which the animal gave up and became immobile—called latency to immobility; the second was the number of bouts of immobility; and finally the third parameter measured was the duration of the immobility when it did occur.

Analysis of the latency to mobility after 10 day of cold stress before the animals were treated with RU-486 was conducted. At this time point, as predicted, the control animals took significantly longer than the stress animals before they gave up and became immobile ($p<0.005$) (Figure 76). Once CORT receptors were blocked for 3 days, the reverse occurred, in that the control animals took significantly less time to become immobile compared to the stress animals ($p<0.0001$) (Figure 76). Interestingly, the EA animals behaved similarly to the control
animals on both days of behavioral testing, in that the difference in time to immobility between the EA and stress animals was significantly lower on day 10 \( (p<0.05) \) and significantly higher than the stress animals on day 13 \( (p<0.05) \).

Bouts of immobility, which was a measure of how often the animal stopped swimming, were analyzed next; and there was no statistical significance between the groups on either pre- or post- RU-486 treatment days (Figure 77).

The unstressed vehicle-control group had a lower duration of immobility, as predicted when compared to the stress group \( (p<0.0001) \) on day 10 of experiment before RU-486 treatments had been started (Figure 78). The EA group’s duration of immobility at this time was also significantly lower than that of the stress group \( (p<0.001) \). After treatment with RU-486, the animals had lower duration of immobility overall. However, the unstressed vehicle-control group had significantly lower \( (p<0.05) \) duration of immobility than the stress group (Figure 78). Interestingly, the EA group had significantly lower duration of immobility compared to the stress group as well \( (p<0.05) \).
Figure 76: Behavioral testing: FST-Latency to immobility pre- and post-treatment with RU-486. On pre- treatment with RU-486 the stress group had significantly higher latency to immobility vs the stress animals. After treatment with RU-486, the control group had significantly lower time to immobility than the stress group (*p<0.01). Interestingly, the EA group behaved similarly to the control animals and had significantly higher latency to immobility when compared to the stress group before RU and lower after RU treatment (♦p<0.05).
Analysis is by one-way ANOVA and post-hoc Tukey's multiple comparison test.
Figure 77: Behavioral testing: FST-Bouts of immobility pre- and post-treatment with RU-486. On pre- and post- treatment with RU-486 days, the control group compared to other groups did not have significantly higher or lower bouts ($p=0.08$ and $p=0.31$, respectively). Analysis is by one-way ANOVA.
Figure 78: Behavioral testing: FST-Duration of immobility pre- and post-treatment with RU-486. On pre-treatment with RU-486 day, the control groups had a significantly lower duration of immobility when compared to the stress group (*p<0.001). On post-treatment day, although the duration was lower for all animals, the control group continued to have statistically lower duration of immobility than the stress group (*p<0.05). The EA group behaved similarly to the control groups on both days in that had significantly lower duration of immobility compared to the stress rats bouts (p<0.001 and p<0.05, respectively).

Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
3.3.2 Open Field Test (OFT)

Several parameters were measured in the OFT. First, the number of sectors visited was counted on both pre- and post- RU-486 treatment days. Next, the number of vertical activity/wall leans was also totaled.

On both pre- and post- treatment with RU-486 days, the control group did not have a significantly higher number of sectors visited (Figure 79). Although the stress animals were the least curious with less visits, as anticipated, this was not significant when compared to the other animals.

For wall leans, when the animal rises up to look over the “fence” as a sign of curiosity, the control, unstressed animals had significantly higher ($p<0.05$) number of wall leans when compared to the stress animals (Figure 80). The EA group also had significantly higher number of wall leans when compared to this stress group ($p<0.05$).
Figure 79: Behavioral testing: OFT-Sectors visited pre- and post-treatment with RU-486. On pre- and post- treatment with RU-486 days, the control group compared to other groups did not have significantly higher visits to various sectors ($p=0.33$ and $p=0.43$, respectively). Analysis is by one-way ANOVA.
**Figure 80: Behavioral testing: OFT-Wall leans pre- and post- treatment with RU-486.** On pre- treatment with RU-486 day, the unstressed control group had significantly higher wall leans compared to the stress group (*p<0.05). On this day, the EA group also had significantly higher leans (♦p<0.05). On the post- treatment day, there was no statistical significance noted amongst the groups (p=0.18).

Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
3.4 Behavioral studies for the propranolol cohort

There were two time points at which the behavioral studies were conducted; one was at 9 days of stress, before the propranolol was administered (Pre-Prop), and the other was on day 13, 5 days after the administration of the propranolol (Post-Prop).

3.4.1 Forced Swim Test (FST)

There were again several parameters measured in the FST. The first was the time at which the animal gave up and became immobile-called latency to immobility; the second was the number of bouts of immobility; and finally the third parameter measured was the duration of the immobility when it did occur.

Analysis of the latency to mobility after 9 day of cold stress before the animals were treated with propranolol was conducted. At this time point the unstressed, control group had a significantly higher time to immobility than the stress group, as well as the sham group (Figure 81). This was not affected by beta blockade with propranolol in that the unstressed, control-vehicle and unstressed control-propranolol groups still had a significantly higher latency to immobility time than the stress group.
The EA group had similar activity as the control groups in that their latency to immobility time was significantly higher than the stress and sham EA groups (Figure 81).

Number of bouts of immobility, which was again, a measure of how often the animal stopped swimming, was also measured in this study group. The unstressed control groups had a significantly lower number of bouts of immobility both on pre- and post-treatment with propranolol days (Figure 82). The EA group also had a significantly lower number of bouts of immobility on both before and after propranolol days than the sham-EA group (Figure 82). When compared to the stress group, by the post-propranolol treatment day, the EA group did not have a significantly lower number of bouts of immobility than the stress group, signifying that perhaps the effect was antagonized by the beta-blocker.

Figure 83 illustrates the duration of immobility the pre-treatment day with propranolol and post-treatment. The control, unstressed group had significantly lower duration of immobility when compared to stress before treatment with propranolol; on the same day the EA group also
had a significantly lower duration of mobility as compared to the stress group.

On post-treatment day, the unstressed control group treated with propranolol had significantly lower duration of immobility when compared to the stress group. Also, interestingly, at this time point the EA animals now had significantly lower duration of immobility than the vehicle-control, unstressed animals.
Figure 81: Behavioral testing: FST-Latency to immobility pre- and post-treatment with Propranolol. On pre- and post- treatment with propranolol days, the control group had significantly higher latency to immobility than the stress group and the sham groups (* $p<0.001$, for both comparison time points).

The EA group’s latency to immobility was also significantly higher than the stress group on both pre- and post- treatment days (♦ $p<0.001$ for both comparison time points).

Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.

* $p<0.001$ vs control groups
♦ $p<0.001$ vs EA
**Pre-Propranolol FST-Bouts of Immobility**

![Bar chart showing number of times immobile](chart1.png)

**Post-Propranolol FST-Bouts of Immobility**

![Bar chart showing number of times immobile](chart2.png)

* * p<0.0001 vs control groups
♦ p<0.05 vs EA

Figure 82: Behavioral testing: FST-Bouts of immobility pre- and post-treatment with Propranolol. On **pre-treatment** with propranolol day, the unstressed, control groups had significantly lower bouts of immobility when compared to stress and sham groups (*p<0.0001). The EA group also had significantly lower bouts of immobility than the stress and sham groups (♦ p<0.05). On the **post-treatment** day, the control animals continued to have statistically significant lower bouts of immobility (*p<0.0001). The same difference was no longer observed in the EA group when compared to the stress group, however, the EA group had significantly lower bouts of immobility when compared to the sham cohort (♦ p<0.05).

Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
Figure 83: Behavioral testing: FST-Duration of immobility pre- and post-treatment with Propranolol. On the pre-treatment day with propranolol, the control, unstressed animals had significantly lower duration of immobility when compared to stress (*p<0.01). At this time point the EA group also had a significantly lower duration of mobility when compared to the stress group (♦p<0.01).

On post-treatment day, the unstressed control group treated with propranolol had significantly lower duration of immobility when compared to the stress and sham-EA groups (*p<0.05). The EA animals had significantly lower duration of immobility than the control, unstressed, vehicle animals (♦p<0.05).

Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
3.4.2. Open Field Test (OFT)

Several parameters were again measured in the OFT with this cohort of animals. First, the number of sectors visited was counted in the animal groups both pre- and post-propranolol treatment. Next, the number of vertical activity/wall leans was also totaled in this cohort.

On pre-treatment with propranolol, the unstressed control groups had a significantly higher number of sectors visited (Figure 84) than all three stressed animal groups, including the EA group. After treatment with propranolol, the unstressed control-vehicle animals had a significantly higher number of visited sectors than the stress group, but not the EA or sham groups.

On the day of pre-propranolol behavioral testing, the control, unstressed animals had significantly higher number of wall leans when compared to the stress, sham and EA animals (Figure 85). After 4 days of propranolol treatment, the unstressed, control-vehicle group had a significantly higher number of wall leans when compared to the stress and sham animals, but this was not observed in the EA cohort (Figure 85).
This suggests that propranolol treatment together with EA may provide an additive protection against anxiety.
Pre-Propranolol OFT-Sectors Visited

Post-Propranolol OFT-Sectors Visited

9 days cold stress

13 days cold stress

* p<0.01 vs control groups

Figure 84: Behavioral testing: OFT-Sectors visited pre- and post-treatment with Propranolol. On pre-treatment with propranolol, the unstressed control groups had a significantly higher number of sectors visited than all the cold-stress groups, including the EA group (*p<0.01). After treatment with propranolol, the unstressed, control group had a significantly higher number of visited sectors compared to the stress groups, but not the sham or EA groups (*p<0.01).
Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
Figure 85: Behavioral testing: OFT-Wall leans pre- and post-treatment with Propranolol. On pre-treatment with propranolol day, the unstressed control groups had significantly higher wall leans compared to the entire cold-stressed animals, including the EA animals (*p<0.001). After propranolol treatment, the unstressed control-vehicle group had a statistically higher number of wall leans when compared to the stress and sham animals but not the EA group (*p<0.05). Analysis is by one-way ANOVA and post-hoc Tukey’s multiple comparison test.
Chapter VI

Discussion
Chronic stress is a major health care dilemma associated with the development of numerous devastating disorders such as cardiovascular disease, cancer, obesity and mental illness. Therefore, discovery of an effective adjunct therapeutic modality to combat stress-induced hormone release would be extremely valuable in addressing the comorbid factors linked to this problem. Hence, the objective was to evaluate the effect of EA on a chronic stress model (cold) and elucidate potential pathways by which this treatment approach may exert its restorative effect. The current results confirm that EA may be a beneficial approach to reducing stress-induced hormones; furthermore, it appears that it exerts its action on the HPA and SNS.

We have organized our discussion in examining EA effects on the HPA followed by SNS effects in the next section.

**EA effect on HPA hormones: CRH, ACTH and CORT**

In order to study the effects of acupuncture on these pathways, a cold stress model as established by Zukowska et al. (1991, 1992, and 1996) was utilized to induce chronic stress [77, 128, 201]. This type of stress stimulates the SNS as well as the HPA axis; it essentially interferes with
the delicate balance of the negative feedback systems such that corticosterone levels remain elevated, and the normal dampening of the stress response is rendered ineffective over time [72]. For example, relative to the SNS, it is well documented that cold stress “highly stimulates” the release of Norepinephrine from sympathetic terminals [72]. However, it is also appreciated that after several weeks of continued cold stress, as in our study, this activity is no longer elevated and the system is dampened i.e., NE levels return to baseline [72].

As the SNS is dampened, with NE returning to baseline, the HPA continues to be activated in chronic stress, such that both ACTH and CRH levels remain high despite elevated corticosterone levels [68, 105, 202-204].

Indeed, our model of chronic stress produced this maladaptive response as demonstrated by our results whereby both circulating ACTH and CORT levels were significantly elevated in rats subjected to stress when compared to control animals. Our stress groups’ ACTH levels followed the above pattern by remaining high in spite of the elevated corticosterone levels. Similarly, in the stress followed by acupuncture group both mean ACTH and glucorticoid levels were not only
significantly lower than the stress alone and sham acupuncture groups, they in fact, approached control animals’ levels by day 14 of treatment. There were no differences noted in ACTH and corticosterone level means between the control animals and the EA animals, as acupuncture had normalized these levels. For example, the mean ACTH level for the control group was 0.27 ng/ml and 0.39 ng/ml for the EA group. In the animals which continued to receive the stress treatments, but not the EA, the levels of these hormones were starting to rise above those animals’ which continued to receive acupuncture. However, the effects remained in that the EA group still had significantly lower levels of both ACTH and CORT ($p<0.01$ and $p<0.05$, respectively) than the stress group.

Interestingly, priming the rats with 4 days of pre-acupuncture treatments, also had analogous effects. It was our hypothesis, that those animals receiving EA before exposure to the stressor would have a better recovery of the plasma and serum HPA hormones in chronic stress. This was indeed the case, in that the EA group in the pre-acupuncture cohort again had significantly lower ACTH and CORT levels than the stress group.
EA also had an impact on central reactivity of the PVN to stress. Immunoreactivity of CRH (CRH-ir) in the parvocellular nuclei of the PVN was significantly reduced in the EA animals when compared to the stress cohort both in the pre- and post-stress EA protocols. This difference was not noted between the EA and control animals, demonstrating that EA had brought CRH-ir down to near control levels. Increased CRH-ir is associated with a variety of stressors as was observed in our model of stress [205-207]. Additionally, PVN CRH mRNA expression was significantly higher in the stress group when compared to the control group.

The HPA axis is controlled by a set of neurons in the medial parvocellular division of the hypothalamic paraventricular nucleus (PVN). The PVN is the focal point of regulation of the interaction amongst the various systems involved in the stress response [208]. There are 20,000 neurons in the rat PVN, which contain more than 20 peptides, neurotransmitters and hormones [208]. Some of these neurons synthesize and secrete CRH, as well as arginine vasopressin (AVP) and oxytocin, which all appear to play an integral role in stimulating ACTH in response to stress [208].
During times of stress, as evidenced in this study, ACTH released from the pituitary, circulates in the blood stream, binds to receptors located in the adrenal cortex, and stimulates cortisol secretion; which under normal conditions helps the organism cope with the stressor. ACTH is also important for its trophic activity on the adrenal cortex such that chronic ACTH stimulation causes an increase in the number of cells, as well as adrenal size [105]. Plasma ACTH levels are under tight negative-feedback control from the glucocorticoids, which are released in response to its secretion. However, during chronic stress cortisol levels remain elevated engendering catabolic consequences that lead to breakdown of vital functions [75].

Elevation in ACTH secretion during stress mirrors CRH secretion patterns [76]. Therefore, as noted above we measured ACTH peripherally and extrapolate the data to understand the role of CRH in our experimental setting. As ACTH stimulates the adrenal cortex to produce corticosterone there is a subsequent rise in glucocorticoid levels during times of stress. This rise in plasma ACTH can be as high as 100-fold increase with a concomitant rise in CORT [209]. With cold exposure, ACTH can rise 1-4 fold over that of unstressed animals as in our model [210].
To further investigate the role of EA on the HPA in this stress paradigm, we utilized RU-486 to block the CORT receptors. In this cohort, by day 14 of stress, plasma ACTH levels, with 4 days of receiving RU-486, were extremely high in the stress group. In fact, the stress group had significantly higher ACTH levels than the EA and control groups. Normally, without pharmacological interference, elevations in CORT lead to eventual lowering of ACTH as part of the protective negative feedback loop. CORT exerts this effect both directly on ACTH release as well as indirectly via decreasing CRH levels. However, by using type II glucocorticoid receptor (GR II) RU-486 we lowered CORT levels, especially in our stress animals, thereby diminishing its effectiveness at providing a negative feedback loop for ACTH, leading to high plasma levels. In fact, the high levels of ACTH noted in our study have been observed by others who have studied the effects of RU-486 on the HPA and, ACTH specifically, in various chronic stress models and are believed to be due to a further dampening of the negative feedback system [211-214].

Based on the above, the effect of EA on ACTH levels was not counteracted by the RU-486. Since the CRH immunoreactivity was
significantly elevated in the EA animals’ PVNs’ dorsal- and medial-parvocellular sections in this cohort versus the stress and sham animals, it may be that EA’s effect on plasma ACTH is via a central mechanism. Perhaps EA had exerted a central effect on CRH levels as evidenced by elevated CRH-ir and had subsequently, decreased ACTH levels despite low CORT levels. Indeed this elevation in CRH was also noted in the control + RU animals’ PVN nuclei sections as a normal, non-stressed reaction to CORT blockade.

The levels of corticosterone itself with RU-486 were very interesting. The CORT levels of all the animals were no longer significantly varied in this RU-486 cohort. CORT receptor blockade had therefore, negated the effect of the stressor as well as EA, and had interfered with fluctuations in serum levels of CORT. Other investigators have also found that pretreatment with RU-486 reduces CORT levels and minimizes variations amongst groups in several stress models [215, 216].

One of the proposed mechanisms for this lowering of CORT with RU-486 treatments is that the drug has a direct effect at decreasing rat adrenal 3β-hydroxysteroid dehydrogenase/isomerase (3-HSD), 21-
hydroxylase and 11-hydrorxylase production which are all involved in glucocorticoid synthesis. Thus, by inhibiting these enzymes, CORT synthesis itself is affected and a reduction in serum levels is observed [217]. Due to this phenomenon, our animals were carefully observed during the cold-stress protocol to ensure that they could survive the stressor. Another explanation for this may be one proposed by Dallman’s groups (2005) that during chronic stress CORT levels are sustained via a positive feed-forward loop. However, once the stress episode is over, systemic CORT and ACTH may be abnormally low [173]. This, together with the RU action on CORT, provides salient reasons for our results in this cohort’s CORT levels.
The stress activated HPA response is coordinated in great part by the SNS. Therefore to examine the role of EA in allaying the stress induced SNS outflow and its influence on the HPA, we blocked the SNS beta receptors. Our rationale was that by blocking the NE receptors EA would no longer be as effective at reducing stress induced HPA activated neurohormones. We utilized propranolol, a peripherally and centrally acting, non-selective beta-blocker. Propranolol is the prototype beta-blocker to which all others are compared and has been used in CV and neurological settings to treat migraines, tremors, anxiety and schizophrenia [218]. It acts presynaptically in the sympathetic neurons at beta 2 receptors, and post-synaptically at beta 1 and beta 2 receptors to decrease NE activity. Its actions included decrease vascular smooth muscle contraction, decreased inotropy and chronotropy of the heart, and anxiolysis, without excessive sedation. Propranolol has also been demonstrated to have other effects outside the classic beta receptor pathways. It can cause a decline in dopamine synthesis, which is a precursor to NE and E as well as interfere with TH activity [218]. Additionally, propranolol can inhibit both NE and dopamine reuptake into synaptosomes of the hypothalamus, and other brain regions such as the striatum [218].
Sympathetic ganglionic blockers could also have been utilized to cause sympathectomy and decrease the SNS outflow associated with stress; however, these agents are all potent sedatives and would have interfered with our behavioral studies as well as our EA treatments altogether. Since our model of EA was undertaken in the awake rat, we believe that the sedative properties associated with these agents would have impaired our assessment of EA mechanisms. In a recent study of EA on the gastric myoelectric activity, sympathetic ganglionic blockers were utilized to examine mechanisms and were associated with moderate sedation [219]. NPY receptor blockers are also available and would have been extremely useful in examining the role of the SNS in its interactions with the HPA as this occurs via Y1R as discussed in the introduction section. However, these agents are extremely costly and it was cost prohibitive to undertake these experiments given our current budget.

In using propranolol we noted that the plasma ACTH levels of the stress group were again significantly higher than the control groups'. However, this difference was not noted in the EA cohort, in fact the EA animals' plasma ACTH levels approached those of the control animals.
Beta-blockade with propranolol had exerted no effect on plasma ACTH as compared to the animals in the previous protocols where the beta receptors were not blocked. The effect of EA on plasma ACTH was therefore not altered by beta blockade.

The propranolol did, however, have direct action on our animals' CORT levels. The stress animals had significantly lower CORT levels that the control group. That there is a great deal of adrenergic and glucocorticoid cross-talk, especially in the CNS, is well described [220, 221]. It has been demonstrated that treatment of neuronal cells in various brain areas with NE and E enhances GR transactivation via beta-2 receptors and that this effect can be blocked with propranolol as well as RU-486 [220]. By blocking beta-2 adrenergic receptors using propranolol, we potentially interfered with the activation of GR. Additionally, as in our study, others have found that beta blockade with propranolol decreases serum CORT levels and that this decrease is more pronounced with greater CORT release under various stress conditions [222-224].

Therefore, in our stress rats, propranolol drove the CORT levels to significantly low levels, whereas EA was able to eliminate this response.
In addition, the EA animals in the propranolol study had significantly lower CRH-ir than the stress animals, similar to the control group animals. Propranolol had, therefore, not interfered with the CRH or ACTH effects of the EA in reducing stress-induced hormones centrally; conversely, beta blockade did affect CORT levels. These results are interesting and indicate that the effects of EA are not altered by beta blockade. An explanation may be that EA’s effect on the HPA is not dependent on SNS activity as it related to the beta adrenergic receptors.

The HPA and ensuing glucocorticoid response to stress have been described by Sapolsky et al. (2000) as modulating, meaning changing the organism’s response to a stressor, or preparative, denoting adjustments in adaptation in response to a chronic stressor [75]. Modulating refers further to a) permissive actions and are present before the stressor and prime the organism to stress; b) suppressive actions which parallel a delayed glucocorticoid reaction which prevents an overshoot of the stress response; and c) stimulating which enhance the effects of the initial response to stress and thus mediate the stress response [75].
From the results we obtained, to apply Sapolsky’s vernacular, it may therefore be concluded that EA not only decreases stress-induced peripheral HPA hormones in a “suppressive” fashion, but that perhaps does so by assisting in the central “preparative” restoration of the negative feedback responses.

It is important to note, that there is no current published data on the effects of acupuncture on peripheral ACTH and corticosterone levels in chronic stress model such as the one we utilized.

There have, however, been a few studies conducted demonstrating the effects of EA on the HPA in acute stress. 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 [159]. They also found that ACTH and corticosterone levels were decreased with EA treatments after toothpulp stimulation was begun [159]. Their animals had similar rise in ACTH and CORT with the stressor which were lowered significantly by EA treatments. In another more recent study of the effects of EA on a painful, acute inflammation rat model, the investigators had similar findings with regards to corticosterone levels which were elevated by acupuncture treatments,
leading to better outcomes in reduction of inflammation and paw-withdrawal latency [225].

We believe the difference in our model and the aforementioned models may be due to varying pathways that exist at other centers in the hypothalamus that are stimulated during electro acupuncture procedures, such as the arcuate nucleus and the periventricular hypothalamic nucleus depending on the type and duration of stress. Indeed, it is well established that there are various pain/stress ascending pathways, as well as, various modulating descending pathways which could have played a role in acupuncture’s specificity in acute versus chronic stress paradigms [172, 226-229]. Such pathways are modulated by a variety of neurally active substances such as nitric oxide, serotonin and acetylcholine [169, 230-232].

In fact, recent studies have demonstrated a direct involvement of ACTH in producing depression and anxiety in chronic anxiety animal models [233]. These models have provided a platform for investigating the interaction of ACTH and serotonergic pathways which seem to be in direct communication and may address the varying responses to chronic versus the more acute stressors discussed above.
Additionally, it is now well understood that both Cannon and Selye’s theories on a non-specific neuroendocrine response despite the nature of the stressor were too generalized [72]. Also, 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 [234]. Stressor-specific responses have been linked to numerous stressors which have been divided into four varying categories, which include a) physical stress such as cold, and pain, such as the one in our model, b) psychological stress, c) social stress and finally d) homeostatic stressors such as hemorrhage, or exercise [72]. 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 studies which have used acute pain/stress as their model.

To summarize our findings with regards to the HPA, we found that EA significantly reduces stress induced hormone levels-ACTH, CORT. Furthermore, that these effects are long lasting, in comparison to pharmacokinetics of agents currently used for anxiolysis, stress
management and depression. EA prior to stress also significantly reduces stress induced HPA hormone activity as evidenced by lowered ACTH and CORT levels. EA also exerted effect on the PVN as it significantly reduced stress-induced PVN CRH expression and immunoreactivity. In our investigation of the mechanism of action of EA on stress induced HPA activation we found that indeed, the expected effects of EA on the HPA are significantly reversed with RU 486, indicating HPA as mechanism. RU significantly reverses effects of stress and EA in the PVN CRH-ir. Finally, to further elucidate the effects of EA on SNS modulation of the HPA we found that the actions of EA are, to a lesser degree, influenced by propranolol possibly indicating a smaller role for SNS beta receptor activity in our chronic stress paradigm.

**EA effect on adrenal and SNS hormones/peptides: NE, TH, NPY**

The importance of the noradrenergic system has also been well established as a classic pathway for responding to stressors in conjunction with the HPA. Numerous investigators have examined the effects of various stressors on NE release specifically in the PVN of the brain [3, 81, 208]. CRH neurons from the PVN project to medullary and
spinal cord autonomic neurons where they regulate the peripheral sympathoneural response to stress [235, 236]. Conversely, the PVN receives catecholaminergic innervation from the medulla oblongata (areas A1, A2 and A5 for NE) and the locus coeruleus [237, 238]. The catecholamines have been demonstrated to synapse on CRH cells in the PVN, and contribute to the alterations in the HPA with stress [80, 239-241].

In fact the highest density of NE terminals in the PVN is found in its parvocellular subdivision that contains most of the CRH-synthesizing neurons [237]. It is however, worth noting that various stressors elicit distinctive responses. For example, although immobilization stress greatly induces c-fos and NE expression in the PVN, cold exposure has been demonstrated to have minimal effect on c-fos and NE expression in the parvocellular PVN especially in chronic cold stress models [242-245]. It is fascinating to discern that after repeated exposure to cold stress, upon treatment with a novel stressor there is an increase in the firing rate of locus coeruleus; and as such the NE system is “primed” to response to this new stressor in the face of chronic stress [133, 246-248].
It has been proposed that cold stress produces an integrated response; however the NE effector loop of the cold stress response has not been identified as yet although several pathways have been implicated [3]. Interestingly, others have reported that cold stress in their analyses have had no effect on PVN CRH expression [249].

Peripherally, sources of NE include sympathetic nerve endings, adrenal medulla and other chromaffin tissue [250]. It is estimated that unlike basal NE, 30-45% of plasma NE in animals is derived from the adrenal medulla during times of stress, although its predominant source under normal and stress conditions is still sympathetic nerve endings [250-252]. This is essential in NE’s primary role in causing vascular smooth muscle contraction and a subsequent redistribution of blood flow to the body’s organs, such as the heart, during times of stress [253, 254].

Numerous studies have described the increase in plasma catecholamines under various stress conditions with several of these stressors, such as immobilization and cold stress causing NE discharge from the sympatho-neural system as described above [72, 78, 81, 133]. However, this can be extremely specific, such that during periods of hypoglycemia, immobilization and emotional stressors adrenal medullary
epinephrine (E) is highly induced; whereas acute cold or pain exposure does not activate E release but causes stimulation of NE release from the sympathetic terminals [72].

Furthermore, in some instances plasma E is increased and in others plasma NE has the greater response. This dissociation of plasma E and NE has been attributed to the specificity of the varying stressors in inducing a sympatho-adrenomedullary response (E) versus sympatho-neural response (NE) [72].

In our stress model, the plasma NE levels in none of the cohorts’ varying groups were significantly different, except in the RU-486 cohort. This could be explained by the above phenomenon that NE levels, although elevated at first, do indeed stabilize over time with exposure to the same stressor [255, 256]. Additionally, the time of collection was after the animals were allowed to acclimate to room temperature for 30 minutes, followed by EA or sham-EA. Given that the elimination half life of NE is 60-90 seconds, the above could have indeed been a factor.

Blockade by RU-486 increased the stress animals’ plasma NE levels significantly when compared to the control animals. It is worth noting
that during the stress response, NE and CRH together increase CORT which in turn reduces NE via negative feedback [257]. Pacak et al., (1995) have also observed that stress increases not only the release but synthesis of NE, which subsequently stimulates CRH release from the hypothalamus PVN [80]. As NE increases, CRH is released, this in turn activates the down stream release of glucocorticoids, CORT [80]. In a classic negative feedback mechanism, CORT subsequently inhibits NE and NE induced CRH [80]. These mechanisms are especially noted in chronic stress models [80]. Indeed, the catecholamines are under the influence of glucocorticoid negative feedback [80]. Since CORT receptor type II was blocked in these animals, the negative feedback loop was hampered, thus the increase in NE levels in this cohort. In a similar stress model in mice, Kuo et al. (2007) found that white adipose tissue (WAT) NE was altered with stress when their animals were fed a high fat and sugar diet (HSHF) [138]. The authors concluded that chronic stress along with a high fat and sugar diet, which increase plasma glucocorticoids, decreases beta adrenergic activity corroborating the feedback system described above [138]. Specifically, in this study Kuo et al. (2007), by using a HSHF diet established an animal model with increased CORT levels [138]. They subsequently found that NE levels were diminished in the abdominal WAT, and attributed this effect as
secondary to prolonged stress and HFHS diet [138]. Indeed this reduction in NE over time (chronic stress) in the presence of prolonged elevated CORT was similarly observed in our study. Furthermore, this effect has also been demonstrated by others who have blocked the glucorticoid synthesis pathway and found that both adrenal and plasma NE levels are significantly increased in response [258]. Interestingly, in this cohort the EA animals had reduced NE levels close to those of the control animals, reflecting a shift towards normalization of NE levels with EA. This effect of EA was maintained despite the decreased CORT levels and stress-induced dampening of the negative feedback system observed in the maladaptive response seen in our stress only animals.

As expected, adrenergic blockade with propranolol decreased the levels of NE peripherally in all animal groups in this cohort, including the stress animals. As noted above, it has been demonstrated that reduced plasma catecholaminergic responses can occur with chronic exposure to the same stressor in a wide variety of stress models [255, 256, 259-263]. Given that NE is produced as a more acute response of “fight or flight”, this may also be a function of acute versus chronic stress models, such as ours.
Nevertheless, several acute human stress studies have demonstrated that EA may modulate the SNS and NE [171, 264]. Wang et al. (2002), found that in their human subjects EA at point Du 20, used in Chinese Medicine for stress release in humans, did affect SNS outflow as measured by stationary R-R intervals on the electrocardiogram [264]. Middlekauff et al. (2002) had similar results, again in a human study. They found that EA inhibited sympathetic activation during acute mental stress in advanced heart failure patients as measured by mean arterial pressure and heart rate [171].

NE is synthesized from L-tyrosine and one of the main enzymes for this reaction is tyrosine hydroxylase (TH), which converts tyrosine to dihydroxyphenylalanine (DOPA). TH is regulated via feedback inhibition by the catecholamines as well as allosteric regulation and phosphorylation by a variety of kinases [72]. TH expression is indeed a major mechanism by which NE and the adrenergic system responds to stress and plays an integral role in adaptation to various stressors [265, 266]. There is in fact an inverse relationship between the two, such that with chronic stress reduced NE response and increased TH activity are noted. This is probably necessary for survival and reflects the
adaptation of the organism to either acclimate to chronic stress, and/or prepare for acute reactions to an urgent stressor [266].

TH protein synthesis, and increased activity as established by increased $K_m$ and $V_{max}$, has been implicated in response to chronic stressors including cold stress [72, 254]. It has also been confirmed that chronic cold stress (over a few days) causes an increase in adrenal TH expression; however, this response is transient and can disappear after several weeks of exposure [72].

It has been well established that TH is increased as a sympathoadrenal response to chronic stressors and can lead to exaggerated response to acute stress in the face of chronic stress [267, 268]. TH increases catecholamines synthesis in the vesicles for release when the organism is faced with an acute stressor. In chronic stress, as NE stores are depleted, TH activity is increased to amplify the stress response as necessary [269]. Chronic stress is also reflected by the activation of the sympathetic-adrenomedullary system, of which TH is an important rate limiting enzyme and has been studied by others in examining chronic stress induced SNS [270].
In our chronic stress model, we were therefore also interested in TH mRNA expression as a biomarker for stress-induced increase in the SNS. Since we found that circulating plasma NE levels as a marker for sympathoneural activity were not altered, we hypothesized that adrenal TH would be increased in response to stress that EA may decrease this stress-induced sympathoadrenal SNS activity. We found adrenal TH mRNA expression levels were increased significantly after 14 days of cold stress in the stress animals as compared to the control animals. In our stress followed by EA protocol the EA animals also had similarly lower mRNA expression of TH as in the control, indicating that EA had decreased sympathetic nervous system activity.

Blocking the GR by RU-486 also had similar effects in that the stress group had significantly elevated adrenal TH mRNA expression as compared to the control groups’ animals. Furthermore, the EA group’s TH levels were significantly reduced, and were close to control animals’ levels. The effects of glucocorticoids, in conjunction with stressors, on TH have been described extensively [78, 257, 271]. It has been well established that endogenous CORT exerts a negative feedback on TH mRNA expression, however, in times of stress, sustained, increased CORT levels actually increases adrenal TH [78, 257]. Conversely, it has
been found that in vivo exogenous treatment with CORT and in vitro treatment of sympathetic neural cells with dexamethasone have both had no significant effects on TH mRNA [78, 138]. However, our model followed a physiologic pattern, in effect, by blocking type II GR receptors, we tampered with the negative feedback system which basal CORT exerts on adrenal TH; thus the significant increases in the adrenal TH mRNA expression of the stress animals versus the controls. EA was able to re-establish this delicate, negative feedback balance between the HPA and SNS in our chronic stress model, leading to lowered adrenal TH mRNA levels despite GR receptor blockade.

As expected, given the inhibitory activity of propranolol not only the adrenergic receptors, but also on catecholamine synthesis, and NE reuptake discussed above, we found the TH mRNA levels in our stress animal animals to be reduced. Although all the animals in the three cold stress experimental groups had some elevation of TH mRNA compared to the control+vehicle group, these slight elevations were not significant in the stress or EA animals, but sham-EA was found to be an added stressor. Sham-EA is discussed in detail in the next sections.
Chronic stress also affects the synthesis and release of numerous neuropeptides such as NPY which are co-localized with the catecholamines, and act as cotransmitters and neuromodulators [111, 137, 138, 253]. Although NE and NPY are co-stored, NE is contained in both small synaptic vesicles and large dense-core ones whereas, NPY is only stored in large ones [72]. Therefore, release of NPY is not congruent with that of NE as control of exocytosis from these two types of vesicles is specific and distinct [272-274]. Additionally, in the rat, platelet NPY also contributes to the levels found in circulation [275, 276]. Therefore, during times of stress, the NPY levels may not parallel NE levels. In our study we examined NPY levels in platelet poor plasma.

Several studies have established that NE levels are elevated during acute response to stress; whereas peripheral NPY levels are not elevated in acute stress [109]. Rather, NPY is increased as a sustained stress response in more chronic situations providing for some resilience in both physical and psychological responses to stress [98, 106, 128, 201]. As such, NPY also has a role in anxiety and behavioral responses to stress partly through the CRH neuronal system [127]. Indeed, elevated NPY expression in the CNS acts as a buffer to the behavioral effects of stress such that it functions as an anxiolytic while CORT and
CRH cause anxiety and depression in chronic stress models [96, 106, 119, 277].

As referred to above, NPY and CRH are interrelated and co-localized in the CNS, and as such work in concert in the stress response. Although, CRH plays the major role of integrating the stress response, there are numerous neural inputs to the PVN that initiate the response. NPYergic neural input from the arcuate nucleus (ARN) is a prominent pathway [114, 278]. Indeed NPY projections are in close proximity with CRH cell bodies and fibers and NPY activity is increased by numerous stressors, including cold stress [113-115, 128, 279].

It has been found that activation of NPY receptors, mainly Y-1, in the PVN stimulates CRH neurons and increases plasma ACTH and CORT, although this is thought to be both dose and time dependent [113, 116, 120, 127]. Acute injection of NPY into the PVN is associated with increases in CRH, with an ensuing elevation in both ACTH and CORT in basal and stress conditions [127, 280]. Conversely, chronic infusion of NPY in the same brain regions has lead to return of basal levels of the above stress hormones in both control and stress animals, indicating the
chronic effect of NPY in inhibition of the HPA rather than its stimulation [127].

It has been further demonstrated that the elevation in CORT in response to chronic NPY injection may be due to negative feedback reduced HPA activity as CORT does exert negative feedback flow to PVN [104, 281]. Inversely, glucocorticoid receptors are found on NPY-containing neurons in the arcuate nucleus, leading to a positive feedback mechanism [104, 282]. As such, the activation of NPY causes increased secretion of CORT which then triggers further release of NPY from the arcuate nucleus leading to the feedforward mechanism thought to be responsible for development of metabolic syndrome [84, 281, 283]. This is largely due to the fact that the ventromedial ARC lacks the blood brain barrier and provides for the direct feedforward communication of CORT on NPY [281].

The NPY results in our model are in concordance with those described by others in that cold stress is a potent activator in the release of NPY into circulating plasma [131, 201, 284]. The stress with acupuncture groups had a significantly lower circulating NPY than the stress group and to a lesser degree lower than those found in the sham acupuncture
group. In fact, in both models of acupuncture, whether pre- or post-stress, NPY was reduced by the EA treatments. Other investigators have examined the role of stress induced NPY in their various EA models. For instance, Lee et al. (2004) found that acupuncture decreased NPY expression in the hypothalamus of rats with Streptozotocin-induced diabetes, which lead to curbing hyperphagia caused by diabetes as compared to diabetic animals which did not receive EA [285]. In another study, Tian et al. (2006) found that EA decreased expression of gastric ghrelin and hypothalamic NPY in chronic food-restricted stress rats when compared to their controls [232].

In our analysis the NPY-ir in the PVN was also increased significantly in our stress animals when compared to the EA and control rats this was also the case for the pre-EA protocol animals. The stress animals also had significantly higher Y1R mRNA expression when compared to the control animals but not the EA group. The pre-EA protocol cohort had similar results in that Y1R mRNA expression tended to be highest in the stress animals when compared to the EA and control group, however, this was not statistically significant., probably due to the variability in qPCR data and the number of animals in each group.
Interestingly the Y2R results were opposite in the two protocols. In the post-EA group, Y2R mRNA expression was not significantly higher in the stress group when compared to the control or EA animals, although these animals had the highest fold induction against GAPDH. In the Pre-EA cohort, Y2R mRNA expression was significantly higher in the stress group when compared to both the control and EA animals. An interpretation of the above results may be that in the post EA animals, the NPY and its Y1R which are upregulated by numerous stressors in the PVN are modulated by the effects of EA and reduced to close to basal levels. The Y1R has been identified as the activating receptor for the action of NPY on the stress-induced HPA activity [113]. It can further be surmised that pre-treatment with EA, induces changes in Y2R mRNA expression similar to control animals whereby levels are brought down to near normal. Given that CORT was significantly increased in the stress animals in these cohorts when compared to the control and EA animals, it may be plausible that this induced the Y2R mRNA expression which was mitigated by pre-EA treatments. Indeed, Kuo et al. (2007) had similar results in observing a significant increase in Y2R mRNA expression of sympathoneural cells treated with the potent exogenous type II GR agonist, dexamethasone [138]. They also found this effect to
be moderated by RU-486, so it may be that pre-EA is working via the CORT pathway and having similar effects as RU-486 on Y2R [138].

Although the perikaryons of NPY are located mainly in the ARC, the PVN the main gathering site for NPY neurons. As previously discussed, these neurons synapse and are in close contact with CRH neurons. NPY is known to have diverse functions in the CNS including regulation of feeding and reproductive behavior, blood pressure, circadian rhythm, and stress response [76]. Stress induced NPY acts as a neurotransmitter and neurohormone. In the CNS numerous stressors alter NPY levels such as the increases seen in immobilization stress in the PVN, or the decreases observed in the ARC and amygdala after chronic immobilization stress [76]. Yet, NPY levels of PVN and ARC were also found to be unaltered by acute restraint (30 minutes) stress [286]. Finally, others have also demonstrated that NPY in the amygdala can rise with repeated immobilization stress and leads to allaying of stress associated anxiety behavior as measured by the elevated plus maze [287]. As discussed above, NPY also coordinated the stress response in the CNS by direct action on activating the HPA and does so via the Y1 receptor [113]. Additionally, NPY, via Y2 receptor in the CNS inhibits release of neurotransmitter and NE presynaptically. Y2 receptors
are the most prevalent receptor type in the CNS and by their action decrease post synaptic excitability [98]. Transgenic mice models have been examined to investigate CNS activity of the Y1 and Y2 receptors. The Y1 knockout animals demonstrate a decrease response to NPY effect on blood pressure, and feeding, where as increased analgesia, body weight, and fat accumulation are all noted [98]. Y2 knockout animals display increase in food intake, body weight and heart rate, but no change in blood pressure and decrease in activity [98]. Therefore the actions of NPY in the CNS are diverse and dependent on the activation of its receptors, type of stressor and area of brain being examined.

In the periphery, NPY is released from sympathetic neurons as well as adrenal medulla and cortex in the rat [288]. NPY also is prevalent in rat platelets. NPY is coreleased with NE in response to numerous stressors and leads to increased vasoconstriction via Y1 receptors. Elevations in NPY plasma concentrations have been observed in various stress paradigms including cold stress, handling, hemorrhage and hypotension [76]. Use of Y1 antagonists have elucidated the mechanism of action of NPY at this receptor peripherally and been used to abate stress-induced rise in blood pressure, and the NPY increase seen with hemorrhage [76].
NPY’s actions in the adrenals is varied and it exists in the medulla and the cortex of rat adrenals and although the content of the medulla is greater, this difference is not large [83]. NPY increases NE release from adrenal capsular tissue via the Y1 receptor but has been shown to have no effect on plasma NPY concentrations despite stress-induced increases in CORT and NE [83, 85]. Release and regulation of adrenal NPY is controlled by splanchnic nerve stimulation which can cause rapid and significant release. Indeed stress is a major activator of adrenal NPY, and various stressors including cold stress have been associated with increases in NPY mRNA [83]. The HPA also has a role in altering the activity of adrenal NPY, ACTH administration causes decrease adrenal NPY in both the medull and cortex; whereas, CORT administration has no effect on basal adreal NPY while it significantly attenuates NPY response to stress [83, 121]. Administration of dexamethasone, a potent glucocorticoid, causes an increase in adrenal NPY and it is believed that NPY is under “inhibitory regulation by glucocorticoids” [83]. In animal studies including the rat, it has been established that NPY has no effect on CORT release, whereas in vitro studies have shown that dexamethasone increases NPY expression in rat adrenals [83].
Since as noted above CORT and NPY have several CNS and peripheral effects on each other to further investigate the role of CORT with NPY in our stress with EA model, we utilized RU-486 to block the GR II receptors. RU-486 reduced the overall levels of plasma NPY in this cohort, especially in the stress and sham-EA animals when compared to the cohort of animals that received the same protocol without RU. One explanation for this is that physiologically, as described above, CORT can increase both central and plasma NPY levels via a feed forward mechanism [84, 173]. By blocking the receptor responsible for this action, we perhaps reduced the neurocircuit positive feedback loop causing overall decreased levels of NPY. CORT also increases NPY release by stimulating GR especially in chronic stress, thus by blocking this receptor we drove the overall levels of NPY down in comparison to the non-RU treated animals in our first experimental protocol. Nevertheless, this maladaptive effect was the greatest in the sham and stress animals, whereas the EA animals’ NPY levels just as in our control group did not decrease in this manner.
This mechanism also explains why the PVN mRNA expression of NPY in this cohort was decreased such that there were no appreciable differences between the control groups’ PVN NPY and the stress groups’ which had received RU and were exposed to cold stress. The observed effect of pre-EA on decreasing NPY mRNA expression was no longer seen after GR blockade, perhaps providing for another mechanism of action of pre-EA on stress-induced NPY mRNA expression. Similarly,
the increase in Y1R and Y2R mRNA expression that were seen in the post- and pre-EA animals was also diminished in this cohort, elucidating a mechanism by which GR blockade had antagonized EA’s noted effects in our previous cohorts. Interestingly, despite RU treatment, the NPY-ir in the PVN continued to be significantly lowered by the EA to close to control levels when compared to the stress and sham animals. An effect of EA on post-transcriptional regulation of NPY in stress may be responsible for this observed discrepancy in mRNA expression versus immunoreactivity at the protein level. This has been reported by others in examining the NPY system and chronic dexamethasone injections into the PVN and arcuate nucleus of the rat brain [289]. The central relationship between NPY and HPA and CORT is certainly complex, especially in the chronic stress arena. The effects of EA on this delicate balance between the two systems observed in this study are certainly fascinating and provoke further investigation.

Adrenal NPY mRNA expression in the RU-486 cohort was noteworthy in that the control+RU animals had significantly higher NPY expression than the stress animals, whereas this was not observed in the EA animals. It seemed that RU 486 had a dual effect, first as a potent indirect activator of NPY by blocking basal serum CORT levels as
observed by high control+RU adrenal NPY mRNA. Next, it seemed to be a persuasive blocker of stress induced activation of CORT levels by minimizing the NPY mRNA expression in the stress+RU animals. However, since the EA animals had similar NPY mRNA to control+vehicle animals, this suggests that EA had conceivably exerted its effect at decreasing RU's receptor blocking ability on the stress-induced elevations in serum CORT and subsequent adrenal NPY mRNA expression. It is also possible that the RU had a direct effect on the animals' adrenal cortex and thus an inter-adrenal effect on NPY mRNA expression. Indeed, although the majority of NPY-ir fibers have been identified in the rat in the adrenal medulla, the rat adrenal cortex has also been demonstrated to possess an extensive number of NPY-ir fibers [83]. Hence, we took the adrenal NPY mRNA content from total adrenal tissue and these inter-adrenal effects could have played a role. Another rationale for our adrenal NPY mRNA results could be that, as previously demonstrated by others, treatment of adrenal medulla with dexamethasone decreases NPY-ir in the adrenals, and further treatment with ACTH increases these levels to near normal levels [121]. Therefore, blocking this pathway with RU together with stress could have caused the above alterations in adrenal NPY content. Additionally, it has been shown in vitro, that rat adrenocortical cells are inhibited by NPY
and as such NPY decreases both ACTH-stimulated and basal CORT levels which in turn decrease NPY via negative-feedback [290]. It is therefore conceivable that by reducing CORT levels with RU, adrenal NPY mRNA expression was increased in the control animals treated with RU.

Since NPY is considered an integral part of the SNS especially in the context of chronic stress response, we explored the role of an adrenergic blocker in further elucidating the effects of EA on this system, particularly NPY. Interestingly, the plasma NPY levels of all the groups were stabilized such that the NPY was no longer significantly lower in the EA group versus the stress animals' levels. In fact, the stress animals' NPY levels were also normalized with propranolol. We had hypothesized that the EA effect would be abolished with an SNS blocker, and indeed the beta blocker prevented NPY levels from significantly varying between groups. Our findings were similar to those established by other investigators who have also found that beta blockade, and propranolol specifically, had profound inhibitory effect on the release of NPY causing a significant decrease in basal release as well as evoked release of the peptide via various stressors [291, 292].
In our pre-EA study EA had significantly decreased stress-induced adrenal NPY mRNA levels in comparison to the stress animals; however, beta blockade with propranolol diminished this effect. Adrenal NPY content is increased by cold stress [131, 284]. Several studies have revealed a bidirectional regulatory role for NPY and catecholamines [85, 108, 112]. NPY causes an activation of adrenal TH and increases the release of catecholamines from the adrenal chromaffin cells via the Y1R [85, 108]. Conversely, NPY has also been shown to inhibit catecholamine release, as well as inhibiting TH especially in some stress models [83, 292, 293]. We have demonstrated above that EA does have an effect on stress induced adrenal NPY content, however once the SNS is blocked, the effects of stress and EA are altogether diminished.

It is again, worth noting, that there are currently no published data on NPY receptor activity, or levels and acupuncture in the chronic cold stress model. However, Lee et al. (2004) found that stimulation at St 36 reduced NPY in both the ARN and PVN of rats with Streptozotocin-induced diabetic rats [285]. Using a food-deprived stress model in the rat, Kim et al. (2001) demonstrated that EA at auricular points reduced the NPY levels in the ARN and PVN of the stress rats while it increased
NPY expression in the control animals [294]. For our study, we also examine the role of propranolol in PVN NPY and its receptors Y1R and Y2R mRNA to determine if beta-adrenergics play a role in the effects of EA on NPY per se. As noted earlier, the NPY mRNA expression and NPY-ir in the PVN were both elevated in the stress animals, and both were significantly reduced by EA. Once we blocked beta receptors the NPY-ir was reduced in all the animals treated with propranolol. Propranolol, in addition to blocking the receptors has a direct effect on inhibiting synaptosomal catecholamine synthesis and uptake in the hypothalamus [218]. Furthermore, the effects of EA observed in stress induced NPY expression in our previous experiments were abolished. Only Y2 receptor mRNA expression was significantly lower in the control animals treated with propranolol when compared to the stress animals. This makes intuitive sense and can be explicated in that Y2R exerts inhibitory effect on catecholamine release thus, decreasing catecholamine activity. Once NE is reduced, Y2R is also decreased due to lack of negative feedback and Y1R is subsequently elevated. Indeed, the Y1R mRNA expression was increased in this experiment, especially in the stress animals when compared to the control and EA animals. However, this was not statistically significant probably due to the normal
variability in the qPCR data and small number of animals' PVN's examined.

In summary, the effects of EA on stress induced SNS activity are as follows, EA whether used as a post-stress or pre-stress treatment significantly reduced stress induced elevation in NPY. However, unlike our observation in the HPA hormones, these effects on plasma NPY levels were not long-lasting. Additonally, EA specifically and significantly decreased adrenal TH; and in the PVN EA significantly reduced stress-induced PVN NPY expression and immunoreactivity. Blockade of the SNS with propranolol abolished the previously observed effects of EA on stress induced NPY levels peripherally, in the adrenals and the PVN. RU-486 had similar effects on mRNA expression of NPY and its receptors in that any effect seen with EA was no longer observed after GR II blockade. Finally, blockade with RU 486 significantly reversed the effects of stress and EA in the PVN CRH-ir; and propranolol significantly reversed EA effects on PVN NPY.
EA Effects on Stress-induced Behavioral Changes

The effects of EA on NPY levels in different brain regions, such as the hippocampus and the amygdala which are both heavily involved with the behavioral response to stress such as depression and anxiety, conflict with the above. In depression and anxiety models EA appears to increase NPY expression in these brain areas which leads to NPY’s stress-allaying effects [295-298]. Indeed, in such models, CRH is clearly viewed as anxiogenic, whereas NPY is considered opposing and anxiolytic.

Given that the above investigators had found a correlation between EA and behavioral changes in various stress conditions; we hypothesized that EA in our model would also decrease the behavioral changes seen with stress such as depression and anxiety. We further hypothesized that by blocking the proposed pathways, SNS and HPA, this effect would be diminished.

In the forced swim test (FST) which is a test for depression and hopelessness, our EA group appeared to be significantly less depressed than the stress group as demonstrated by longer times to immobility as
well as lower durations of immobility. Before the RU-486 treatment, in the latency to immobility test, which signifies hopelessness, depression and in a sense “giving up”, the stress animals gave up significantly faster than the EA animals which behaved similarly to the control group. After CORT- receptor blockade, however, the stress animals swam significantly more than the control animals and EA animals. We believe this to be caused by the significant increase in the adrenal TH levels of the stress group when compared to the control and EA animals. The stress animals probably had an exaggerated response to the FST as an added acute stressor and swam wildly. In other words, the chronic stress paradigm here had primed them for an overwhelming response to the acute stress of the FST. EA was able to allay this response as seen above in significantly lowering TH adrenal levels and decrease latency to immobility similar to those of control animals. Given the differences in the behavior of the animals’ pre- vs post-RU-486, we believe that this further validates that EA exerts its effects in this paradigm via peripheral CORT pathways. Studies in mammals and humans have shown that there is an increase in serum CORT levels in depression and anxiety, further demonstrating that CORT and CRH are both anxiogenic peripherally and centrally respectively [119, 299, 300]. Our results are similar to other investigators who have found that EA affects the HPA,
and CORT and may affect the ensuing behavioral changes related to it serum levels [298, 301, 302].

Blockade by propranolol did not affect EA’s ability to increase the latency to immobility to similar to that of the control group which was significantly longer than the stress group. However, the bouts of immobility and duration of immobility which were at first similar in the EA and control groups as above, were affected by blockade with propranolol. The bouts of immobility were no longer significantly lower than that of the stress group; also the duration of immobility was similar to that of the stress group and significantly higher than the control group. It can be concluded that EA may affect the depression caused by stress via the SNS pathway since blockade with propranolol had significant consequence in diminishing EA’s ability to allay these effects. Several studies have confirmed that EA alters catecholamines peripherally and centrally, and that these effects may lead to EA as a useful adjunct therapy to anti-depressants and anxiolytic treatments [303-308].

In the open field test (OFT), which we utilized to examine the anxiety behavior related to stress the animals did not behave differently with regards to sectors visited. However, the EA group had similar activity to
the control group in that the animals were significantly more curious and less anxious as noted by the number of wall leans when compared to their stress alone counterparts. This effect was however, diminished by RU-486, providing further evidence that EA may reduce anxiety caused by chronic stress and elevated CORT levels.

For the propranolol group, in the OFT test, the EA animals visited more sectors but had similar wall leans compared to the stress animals pre-treatment, however, they were still significantly more anxious than the control group. The control group continued to have significantly higher visits and leans than the stress with propranolol group after treatment. However, upon treatment with propranolol the EA group visited similar numbers of sectors as the control-vehicle group and had no significant difference in wall leans as this group. This may provide more evidence that perhaps EA and beta blockade had added effectiveness in relieving anxiety related to stress. Other investigators have suggested similar effects of EA and anxiety. Vickland, et al. (2009) investigated the role of EA in anxiety and sympathetic outflow and found that EA had “counterbalanced” anxiety related sympathetic outflow as measured by heart rate variability in their human subjects [309].
In a systemic literature review, Pilkington et al. (2007) investigated the function of acupuncture for anxiety, and found that although positive findings are promising, there is currently a lack of scientific evidence to draw any conclusions [310].

Taken together, the above results indicate that acupuncture has a reducing effect on the circulating neurohormones and neuropeptide Y which are involved in an organism’s reactions to chronic stress. EA also had a significant effect on reducing TH and NPY in some of the experiments, elucidating an integrated peripheral action together with the adrenals. Additionally, acupuncture significantly reduced the expression of CRH and NPY in the PVN as well as modulated the NPY receptors responsible for the coordination of the stress response in the PVN.
Target Tissues

++ACTH
++CORT
++NPY/++NE
++CRH

CHRONIC STRESS EFFECTS
??BP/HR
-/+Feeding
- - Reproduction
- - Immunity
- - Growth/Thyroid Function

CHRONIC STRESS EFFECTS with EA ABATED DUE TO:
↓ CRH/NPY IN PVN
↓ TH/NPY IN ADRENALS
↓ ACTH/CORT/NPY IN PLASMA

PVN
CRH
NE
NPY

Locus coeruleus
++NE

Sympathetic preganglionic neurons
++NE

Sympathetic ganglia

Chronic stress
--

Acupuncture

Central

+ +

Adrenal

--

Plasma

Target Tissues

++NPY/++NE
++CORT

++CRH

++ACTH

Arcuate nucleus
++NPY
Our findings in the sham acupuncture group levels of neurohormones are most intriguing. Sham acupuncture increased NPY levels beyond that of the stress group, behaving as an added stressor; yet decreased ACTH and corticosterone although not as effectively as EA. In most of the experiments we conducted, however, sham EA seemed to elicit an additive stress response to the cold. This could have been due to the location of the sham and that it may have caused the animal some discomfort. However, choosing a sham point in an awake rat posed some technical challenges. The point selected had to meet two basic criteria that, a) the awake, freely moving animal not be able to reach the needles for the duration of the treatment and b) they be located at a far enough distance from active, EA points in the rat; which given the small surface area of the animal posed some challenges.

There have been many discussions, of late, with regards to sham acupuncture [311-313]. The unforeseen effects of sham acupuncture are well documented [314, 315]. In humans the effects of sham are quite varied and unpredictable, although with the aid of fMRI technology, we can at least begin to appreciate the central pathways elicited by sham versus verum EA.
In a human functional MRI study Qin, et al. (2008), found that both sham and EA acupuncture had a physiologic effect on various brain regions [316]. Compared to acupuncture, sham presented increased connectivity among certain brain regions especially those involved in modulation of pain. They concluded that this was caused by more intense sensations commonly induced by sham stimulation in humans [316].

In another study, the effects of true and placebo acupuncture were examined in human subjects by quantitative electroencephalography (qEEG) and heart rate variability (HRV) [38]. The investigators found that EA acupuncture caused significant activity in the fast EEG frequency whereas placebo acupuncture did not elicit the same response. In addition, they inferred that true acupuncture had a stress-reducing effect by induction of specific central and autonomic nervous system activity, whereas sham acupuncture activated the nociceptors [38].

The animals in our sham acupuncture group had increased levels of neurohormones associated with stress. Although by day 14, sham acupuncture had exerted an effect in reducing the levels, it was not as
effective as true acupuncture in lowering the levels to near control groups’. As conferred above, sham acupuncture has indeed been deemed to have effect, and our study is congruent with these findings. As previously mentioned, sham acupuncture in our model, similar to others, had numerous unpredictable effects, and at times behaved as an added stressor. Sham-EA animals in our paradigm behaved similarly to EA in the first cohort, albeit was not as effective. More consistently, however, sham-EA in our experiments was also an activator of central and peripheral neurohormones involved in stress. Centrally, sham EA animals consistently demonstrated both elevated CRH-ir and NPY-ir which were similar to stress animals’ and significantly higher than the animals treated with EA. Hence we believe that sham-EA, at least in our model, was itself a stressor and provided very few, if any, of the benefits of EA seen in the verum EA groups. This has been corroborated by others. In another fMRI study, Wu et al. (2002) found that there was indeed neural specificity with regards to EA versus sham EA [44]. They found that EA at St 36 modulated the hypothalamic-limbic system, whereas sham EA in their model did not. Conversely, they found that both sham and EA had similar effects on the visual and auditory cortices [44].
In conclusion the above results indicate that EA has the ability to significantly decrease stress induced neurohormones and neurotransmitters of the HPA and SNS in the PVN, adrenal glands and peripherally as reflected in circulating levels. The mechanism by which EA exerted these effects on the HPA and SNS was further elucidated by using GR II blockade and beta blockade, respectively. It was found that previously significant decreases in stress induced elevation exerted by EA was significantly reversed with the above blockers, especially RU-486, indicating further evidence that EA exerts its stress allaying effects via the HPA and SNS as was hypothesized.

Given the vast number of debilitating diseases linked with stress, it is extremely important and of great significance to public health to assess the modulation of stress pathways. Tapping into a body of knowledge, TCM, that provides a different paradigm in addressing this disease-linked issue is therefore extremely significant. Acupuncture may provide a cost-effective, relatively non-invasive, and non-pharmacologic treatment modality. The extrapolation of the above data to populations, and the implication of positive results in humans suffering from chronic stress would provide utility in addressing this major health dilemma. Certainly, the results are intriguing and merit further investigation.
**Limitations of the study**

There were several limitations to our study. Although power analysis had been performed based on our pilot data, due to the fact that we used half of the brain samples for IHC and half for qPCR we found that our sample size was a factor in achieving power and significance in some of our qPCR data. An increase in the sample size would address this issue; however, since we only had one acupuncturist conducting sham and EA treatments an increase in our sample size would have provided other challenges such as altering the time for blood draws after EA treatments. In the future conducting these experiments with more animals, and acupuncture practitioners would be of value.

We utilized propranolol as our SNS activity blocker, but others as discussed below in future studies section, may provide a better venue for investigating the role of SNS in stress allaying effects of EA. Additionally, our animals were housed at the animal facilities at Georgetown University, which during this set of experiments was being painted and may have impacted the animals’ response to the stressor and the sham and EA treatments.
**Proposed future studies**

To further elucidate the role of Y1 Y2 receptors in the effects of EA on stress induced NPY activity, it would be of great interest to utilize the above paradigm with the addition of Y1 and Y2 receptor antagonists.

Also to further assess the effects EA would produce in an over active NPY state as observed in stress, it would be of interest to investigate its effects in transgenic rats which overexpress NPY.

Use of alpha 2 agonist or a sympathetic ganglionic blocker would also be of great value in examining the effects of EA in decreasing stress via the SNS. These drugs can provide for further evaluation of the SNS in EA’s effects on the SNS as an anxiolytic.

There is a large body of evidence, as discussed in the introduction section, which demonstrate that the opioid system is activated in EA’s ability to decrease pain and acute stress. Our stress paradigm may have overlap with the opioid system given that the animals probably had pain after an hour on ice. Additionally, it was hypothesized that the sham EA point may have caused discomfort and pain. It would therefore be constructive to examine the opioid system in this model and further
elucidate its effects on this important system by using an opioid antagonist such as naloxone.

Another system involved in the anxiolytic pathway as it relates to chronic stress models is the activation of the GABAnergic system. Therefore examining EA activity on GABAnergic pathway in our stress model may provide for an additional mechanism by which EA exerts its effects.

Finally, a natural antagonist of CRH, oxytocin, may have a role in EA’s ability to decrease stress induced responses in the PVN. Hence, future studies using this paradigm in examining PVN oxytocin may provide an additional venue by which EA may exert its effect by recruiting this naturally occurring PVN antagonist to CRH.


190. Marks, W., N.M. Fournier, and L.E. Kalynchuk, Repeated exposure to corticosterone increases depression-like behavior in two different versions of


World Wide Web references:


