Role of noradrenergic signaling in the basolateral amygdala in habituation to repeated stress

by

Nicola M. Grissom

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Psychology)
in The University of Michigan
2009

Doctoral Committee:

Professor Theresa M. Lee, Co-chair
Assistant Professor Seema Bhatnagar, Co-chair, University of Pennsylvania
Professor Stephen A. Maren
Assistant Professor Gina R. Poe

Dedication

This accomplishment is dedicated to my grandmother, Dr. Doris Jasinski, who would not have been surprised.

Acknowledgements

First, I thank my advisor Dr. Seema Bhatnagar, who has supported me in so many ways throughout my time in her lab. It goes without saying that this would not have been possible without her, in more ways than I can name. An example: my projects and publications have been "just a little different" from the rest of the field, and I am so grateful to her for her willingness to pursue these ideas with me. Another: she let me learn by doing, which is really the only way to learn, but never left me without feedback.

Many people in the lab have provided technical assistance with my experiments, and I thank them all. In particular, though, I would like to express my gratitude to Vikram Iyer, Kai Denski, Courtenay "Larry" Vining, and Sandra Luz, and out of all of my student assistants, Sumeet Goel and Janani Singaravelu. These individuals, from both UM and Penn, provided excellent technical assistance, but more importantly they made surgery and sample collection as fun as humanly possible.

In addition, many scientists outside of our laboratory have provided intellectual and/or technical assistance concerning the experiments in this dissertation. I thank the members of my committee, Drs. Theresa Lee, Stephen Maren, and Gina Poe, for their guidance and patience. In addition, I am grateful to the technical assistance of Dr. Rita Valentino, her postdoc Dr. Debra Bangasser, and Dr. Ted Abel. These individuals assisted me in learning the Western blot technique used in Chapter 4, which significantly extended the findings of this dissertation. I also want to note that the results in the Appendix were previously published as: Grissom, Kerr, and Bhatnagar. Struggling behavior during restraint is regulated by stress experience. *Behav Brain Res.* 2008, 191(2).

Lastly, I want to thank my family, especially my aunt Sara Grissom, who took a chance on moving to Michigan from Oregon, and my husband Christopher Herdt, who took a chance on moving to Philadelphia from Michigan. Without them, I wouldn't be where I am today, and not only because I needed someone to drive the U-Haul. They are my greatest cheerleaders.

Table of Contents

Dedication ii
Acknowledgements iii
List of Figures vi
List of Tables xiv
List of Abbreviations xv
Abstract xvi
Chapter 1. Introduction 1
1.1 Overall introduction 1
1.2 Stress responsive systems 2
1.3 Neural regulation of adaptive responses to repeated stress 9
1.4 The BLA in aversive learning 11
1.5 Overall hypothesis and specific aims 14
Chapter 2. β−AR signaling in the BLA bidirectionally modifies the habituation of HPA and behavioral responses to repeated restraint 18
2.1 Introduction 18
2.2 Methods 21
2.3 Results 30
2.4 Discussion 39
Chapter 3. Gene expression in stress-regulatory brain regions is altered by repeated restraint and intra-BLA β−AR blockade 46
3.1 Introduction 46
3.2 Methods 48
3.3 Results 51
3.4 Discussion 55

Chapter 4. Intracellular signaling in the BLA regulates HPA activity to restraint..... 61
4.1 Introduction 61
4.2 Methods 64
4.3 Results 70
4.4 Discussion 75
Chapter 5. Discussion 84
5.1 Overview 84
5.2 Major findings 85
5.3 Implications of current findings in light of broader literature 89
Appendix A. Struggling behavior during restraint is regulated by stress experience. 104
A.1 Introduction 104
A.2 Methods 106
A.3 Results 113
A.4 Discussion 121
References 127

List of Figures

Figure 1.1 Schematic of the HPA axis. Stress activates the PVN (hypothalamus) to secrete the neuropeptides CRF and AVP. These peptides stimulate the anterior pitutiary to synthesize and release ACTH. ACTH travels through general circulation to the adrenal gland, located above the kidney, and stimulates the adrenal cortex to synthesize and release glucocorticoids (corticosterone in rodents). Glucocorticoids exert wide ranging physiological effects to enable the organism to escape or challenge the stressor at hand. Glucocorticoids also provide negative feedback at the level of the brain and pituitary to inhibit further HPA activity. See text for further details. 3

Figure 1.2 Summary of McGaugh model of β-AR function in the BLA. Taken from Roozendaal, Schelling, and McGaugh, 2008. In this model β-AR constitute the primary mechanism by which NE modulates memory consolidation. The actions of glucocorticoids, acting via GR, of CRF acting through the CRF-1 receptor, and of α1-AR stimulation is to enhance the actions of β-AR signaling to PKA. α2-AR are depicted as solely presynaptic autoreceptors which inhibit NE release into the synaptic cleft. PKA activation via cAMP is thought to underlie the modulation of memory consolidation, though the exact mechanisms are unclear. 13

Figure 2.1 Experimental design for Experiment 2.1. After recovery from the implantation of intra-BLA cannulae, animals were either restrained for 30 min or remained in the home cage on days 1-4. Some animals which had been restrained (RR VEH and PROP) received intra-BLA injections immediately following daily restraint. Home cage animals received intra-BLA injections at this time. The remaining repeatedly restrained animals received intra-BLA injections 4 hours following the end of restraint (RR DELAY). On day 5, no drug was administered and all animals were restrained for 30 minutes and had blood samples taken at the times indicated. The design of subsequent experiments in this dissertation is based on this design. 24

Figure 2.2 Cannulae placements in the BLA. In (a) is an example of a cannula directed at the BLA. In (b) is a plate from Paxinos and Watson (2004) depicting the BLA and surrounding structures at the same approximate level (Bregma -3.3) as in (a) and (c). In (c) is an example of a cannulae which has missed the BLA. The BLA is outlined in (a), (b), and (c) based on the depiction of the nucleus in (b)..... 30

Figure 2.3 HPA activity to restraint after repeated restraint and intra-BLA propranolol in Experiment 2.1. In (a) and (b) are ACTH values for restraint on day 5, and in (c) and (d) are corticosterone values. The six groups are divided between two graphs for clarity, and RR VEH and RR PROP are included in both for comparsion. (a) Compared to CTL VEH, RR VEH is significantly (*) habituated at 15 minutes, and trends ($p \leq 0.06$) towards significant habituation at 30 minutes. Compared to RR PROP, RR VEH is significantly habituated at 15 and 30 minutes. (b) Compared to RR PROP, all other groups in this graph are significantly habituated at 15and 30 minutes into restraint. These results indicate that immediate post-restraint propranolol into the BLA on days 1-4 prevented the habituation of ACTH activity on day 5 in RR PROP animals, but that delayed post-restraint administration of propranolol did not prevent habituation in RR DELAY PROP animals. There were no significant effects of corticosterone in (c) and (d). Please see text for full list of significant comparisons. Figures are means ± SEM. 33

Figure 2.4 Struggling during restraint after repeated restraint and intra-BLA propranolol in Experiment 2.2. Figures (a) and (b) depict strong mobility, (c) and (d) depict light mobility, and (e) and (f) depict immobility. Figures (a), (c), and (e) depict these measures in 5 minute bins across the 30 minute restraint on day 5, and (b), (d), and (f) depict the total time spent in these behaviors. In (a), compared to all other groups, RR VEH animals show a significant (*) habituation of strong mobility (the struggling response). This indicates that intra-BLA propranolol prevented the habituation of struggling behavior in RR PROP animals. See text for full list of significant comparisons. Figures depict means ± SEM. 36

Figure 2.5 ACTH activity to restraint after repeated restraint and intra-BLA clenbuterol in Experiment 2.3. In (a) are ACTH concentration for RR groups only at the end of restraint on day 3. In (b), (c), and (d) are ACTH concentration in response to restraint on

day 5. CTL VEH and RR VEH groups are included on all three graphs for comparison. In (a), 1ng clenbuterol after restraint on days 1 and 2 resulted in a significant (*) reduction in ACTH concentrations at the end of day 3 compared to vehicle. The 3ng dose tended to be lower than vehicle on day 3 (+, $p \leq 0.06$). At the end of 30 min restraint on day 5 in (b), comparison of the repeatedly restrained groups indicated that the 1ng dose of clenbuterol (RR 1ng) resulted in reduced ACTH concentration compared to repeatedly restrained animals which received vehicle after daily restraint (RR VEH). In sum, 1ng clenbuterol in the BLA after daily restraint enhanced habituation to restraint. See text for a full list of significant comparsions. Figures depict means ± SEM. 38

Figure 2.6 Results of missed BLA placements in Experiment 2.1. Those animals in the RR PROP group which did not have cannulae directed at the BLA were excluded from analysis. These excluded animals, collected as the MISSED RR PROP group, demonstrate habituation of ACTH concentrations in response to restraint on day 5. Figure depicts means ± SEM. 44

Figure 3.1 AVP mRNA in the PVN after repeated restraint and intra-BLA propranolol. In (a) is a depiction of the PVN and surrouding structures, and the medial parvocellular PVN, where AVP and CRF was measured, is indicated. In (b) is example PVN from the groups in this study, showing increased AVP mRNA density in the PVN of repeatedly restrained animals that received vehicle, but not propranolol, after daily restraint. In (c) is the graph of AVP mRNA density. RR VEH animals showed significant elevations (*) in AVP compared to CTL VEH and RR PROP, and tended (+, $p \leq 0.12$) to show increased AVP mRNA density compared to CTL PROP. (c) depicts means ± SEM. 52

Figure 3.2 CRF mRNA in the PVN after repeated restraint and intra-BLA propranolol. In (a) is a depiction of the PVN and surrounding structures as in Figure 3.1(a). In (b) is an example of CRF mRNA density in each group. In (c) is a graph of CRF mRNA density in this experiment. No significant effects were seen in this measure. Figure depicts mean ± SEM. 53

Figure 3.3 BDNF mRNA in the BLA after repeated restraint and intra-BLA propranolol. In (a) is a depiction of the BLA with the lateral and basal nuclei delineated. In (b) are example BLA from the groups in this study, depicting the increased BDNF mRNA in the

basal nucleus of the BLA of repeatedly restrained rats given post-restraint vehicle, but not propranolol. In (c) and (d) are graphs of BDNF mRNA density in the basal (c) and lateral (d) nuclei. BDNF mRNA in the basal nucleus of RR VEH animals is significantly (*) higher than in all other groups. No differences were seen in BDNF mRNA density in the lateral nucleus. Graphs are means ± SEM. 54

Figure 3.4 BDNF mRNA in the hippocampus after repeated restraint and intra-BLA propranolol. In (a) is a depiction of the hippocampus with the areas of interest demarcated. In (b) are representative hippocampi from groups in this experiment. In (c), (d), (e), and (f) are graphs of mean BDNF mRNA density in the dentate gyrus (c), CA3 (d), CA2 (e), and CA1 (f). In (c), BDNF mRNA in the dentate gyrus of CTL PROP, RR VEH, and RR PROP animals was significantly (*) elevated compared to CTL VEH. In (d), BDNF mRNA was significantly elevated in repeatedly stressed groups relative to controls. There were no effects in CA2 or CA1. Graphs are means ±SEM. 55

Figure 4.1 Diagram of intracellular signaling mechanisms discussed in Chapter 4. β-AR is classically thought to act via cAMP/PKA mechanisms, one important action of which is CREB phosphorylation, which leads to increased histone acetylation (AcH3). β-AR may also phosphorylate ERK (pERK), which may also activate CREB. pERK and AcH3, which were explicitly measured in this chapter, are emphasized. See text for further details. 64

Figure 4.2 BLA pERK and AcH3 after no stress, a single restraint, or repeated restraint in Experiment 4.1. (a) is an example Western blot for pERK and total ERK in this experiment. (b) depicts pERK1/ERK1 and c depicts pERK2/ERK2. (e) is an example western blot for AcH3 and total H3 in this experiment. (f) depicts AcH3:H3. pERK1/2 in (b) and (c) were significantly (*) reduced in the BLA of both CTL and RR animals at the end of restraint as compared to a basal animal (US). AcH3 was significantly increased in the BLA of CTL animals at the end of a first 30 minute restraint as compared to basal US animals. RR animals at the end of a 5th restraint were not different from either US or CTL animals. Thus, acute stress reduces pERK and increases AcH3. Figures are mean ± SEM..... 71

Figure 4.3 Hippocampus pERK and AcH3 after no stress, a single restraint, or repeated restraint in Experiment 4.1. (a) is a representative Western blot for pERK and total ERK in this experiment. (b) depicts pERK1/ERK1 and (c) depicts pERK2/ERK2. (e) is a representative Western blot for AcH3 and total H3 in this experiment. (f) depicts AcH3:H3. No significant differences were seen in pERK or AcH3 in the hippocampus. Figures depict means ± SEM. 72

Figure 4.4 BLA pERK and AcH3 in animals sacrificed basally after prior repeated restraint and intra-BLA propranolol in Experiment 4.2. In this experiment, all animals were sacrificed basally on day 5. Thus all differences are tonic changes induced by prior restraint and intra-BLA drug administration. (a) is a representative Western blot for pERK and total ERK in this experiment. (b) depicts pERK1/ERK1 and (c) depicts pERK2/ERK2. (d) is a representative Western blot for AcH3 and total H3 in this experiment. (e) depicts AcH3:H3. pERK1/2 in (b) and (c) was significantly (*) reduced in the BLA of repeatedly restrained animals given intra-BLA vehicle after daily restraint (RR VEH) as compared to unstressed animals which received intra-BLA vehicle (CTL VEH) or repeatedly restrained animals given intra-BLA propranolol (RR PROP). The difference between RR VEH and CTL PROP tended towards significance (+, $p = 0.09$). AcH3 in (e) was significantly increased in the BLA of RR VEH animals as compared to CTL VEH, and tended ($p = 0.08$) to be increased compared to RR PROP. Thus, propranolol in the BLA after daily restraint attenuates intracellular signaling changes in the BLA induced by repeated restraint. See text for complete results. Figures depict means ± SEM. 73

Figure 4.5 Hippocampus pERK and AcH3 in animals sacrificed basally after prior repeated restraint aind intra-BLA propranolol in Experiment 4.2. In this experiment, all animals were sacrificed basally on day 5. Thus all differences are tonic changes induced by prior restraint and intra-BLA drug administration. (a) is a representative Western blot for pERK and total ERK in this experiment. (b) depicts pERK1/ERK1 and (c) depicts pERK2/ERK2. (d) is a representative Western blot for AcH3 and total H3 in this experiment. (e) depicts AcH3:H3. No significant differences were seen in pERK or AcH3 in the hippocampus. Figures depict means ± SEM. 74

Figure 4.6 ACTH activity in response to restraint after repeated restraint and MEK inhibition in the BLA in Experiment 4.3. (a) depicts the ACTH response to restraint on day 5, and (b) depicts the integrated values for the ACTH response in 4.5(a). In (a), repeatedly restrained (RR) animals had significantly (*) reduced ACTH concentrations in comparison to CTL animals at 15 and 30 minutes into restraint, and animals which received intra-BLA MEK inhibitor (MEKI) tended (+, $p \leq 0.09$) to have reduced ACTH concentratins in comparison to VEH animals. In (b), RR significantly reduced integrated ACTH concentration in comparison to CTL, and MEKI significantly reduced ACTH concentrations in comparsion to VEH. Figures depict means ± SEM. 75

Figure 5.1 McGaugh and Roozendaal model of β−AR actions in the BLA. From Roozendaal, Schelling, and McGaugh, 2008. In this model, cAMP activation by β−AR is the crucial step mediating the effects of β−AR stimulation. It does not account for the overall inhibition of neurons by NE as demonstrated by Buffalari and Grace (2007, 2009), or for the reduction in pERK in the BLA associated with β-AR activation in repeatedly restrained rats as seen in Chapter 4. See text for more details. 90

Figure 5.2 Revised model of β-AR actions in the BLA. It includes the hypothesized competitive relationship between PKA and ERK effects in the cell suggested by the results of Chapter 4, and as described in this chapter. CRF, GR, and α-AR effects from Figure 5.1 were not included to simplify this figure, though they are still presumed to occur. pERK is known to increase HPA activity as inhibiting pERK decreases HPA activity (Chapter 4). Histone acetylation is presumed to decrease HPA activity as it is increased in the BLA of repeatedly stressed animals that did not receive β-AR blockade (Chapter 4). See text for further detail. 92

Figure 5.3 Model of the effects of propranolol administration in PTSD patients. Propranolol has been shown to reduce PTSD symptoms when given in conjunction with the trauma or reactivation of memory for the trauma, without causing amnesia for the trauma. PTSD can be thought of as a lack of habituation to the stressful re-experience of memories of the trauma. PTSD patients given propranolol may be better able to habituate as propranolol reduces β-AR agonist activity in the BLA, which may be hyperactive in these individuals as compared to nonpathological individuals exposed to

trauma. However, increasing doses of propranolol should no longer be effective at reducing PTSD symptoms as it pushes individuals further left on the dose-response curve, reducing habituation. See text for details. 103

Figure A.1 In Experiment 1, animals were restrained on day 5 with (repeated restraint group) or without (acute restraint group) 4 prior days of repeated restraint stress. (A) Graphs show total time spent immobile, lightly mobile, and strongly mobile (struggling) during 30 min restraint on day 5. (B) Timecourse of time spent strongly mobile (struggling) across 30 min restraint on day 5. All data are expressed as mean ± S.E.M. Asterisks indicate repeated restraint group values significantly different from acute restraint group values. 113

Figure A.2 In Experiment 2, animals were restrained on day 5 after 4 days of repeated forced swim (swim + acute restraint group) or without prior swim (acute restraint group). (A) Graphs show total time spent immobile, lightly mobile, and strongly mobile (struggling) during 30 min restraint on day 5 in animals. (B) Timecourse of time spent strongly mobile (struggling) across 30 min restraint on day 5. All data are expressed as mean ± S.E.M. Asterisk indicates that acutely restrained rats exhibited higher strong mobility than acutely restrained rats after swim throughout the 30 min period of testing. 114

Figure A.3 In Experiment A.3 animals were first sham operated (sham) or adrenalectomized (ADX) and replaced with subcutaneous corticosterone pellets prior to 8 days repeated restraint. (A) Graphs show total time spent immobile, lightly mobile, and strongly mobile (struggling) in both groups on days 1 (acute restraint), 5, and 8 of restraint. (B) Timecourse of time spent strongly mobile (struggling) across 30 min restraint on days 1, 5, and 8, divided by surgery. All data are expressed as mean ± S.E.M. Asterisks indicate repeated restraint group values significantly different from overall acute restraint group values. 116

Figure A.4 In Experiment A.4 animals were first sham operated (sham) or adrenalectomized (ADX) and replaced with subcutaneous corticosterone pellets, then were either undisturbed until restraint on day 5 (acute restraint) or given 4 days of repeated forced swim prior to restraint on day 5 (swim + acute restraint). (A) Graphs

show total time spent immobile, lightly mobile, and strongly mobile (struggling) in sham and ADX animals during 30 min restraint on day 5. (B) Timecourse of time spent strongly mobile (struggling) across 30 min restraint on day 5, divided by both stress history and by surgery. All data are expressed as mean ± S.E.M. Asterisks indicate overall repeated swim + restraint group values significantly different from overall acute restraint values. 118

Figure A.5 (A) In Experiment 5, animals were placed in forced swim for 15 min with (rep. restraint + swim group) or without (acute swim group) 7 prior days of repeated restraint. (A) Graphs show total distance traveled during 15 min forced swim on day 8. (B) Timecourse of times spent immobile, lightly mobile, and strongly mobile across 15 min swim on day 8. All data are expressed as mean ± S.E.M. 120

List of Tables

Table 4.1 Summary of findings in the BLA from Experiments 4.1 and 4.2. The degree of change in each group of both experiments is in reference to the levels of pERK and AcH3 in the BLA of the groups marked with an asterisk (*). The US group in Experiment 4.1 and the CTL VEH group in Experiment 4.2 can be considered equivalent as neither has received drug treatment or any stress prior to tissue collection. The cell marked with a dash (no difference) and an arrow indicate that there was a trend for this change to have been prevented in the RR PROP group..... 77

Table A.1 Plasma ACTH and corticosterone levels collected at the end of 30 min restraint on day 5 (Experiments 1, 2, and 4) or day 8 (Experiment 3). In Experiments 1 and 2, animals were acutely restrained on day 5 with or without 4 days prior repeated restraint (Experiment 1) or repeated swim (Experiment 2). In Experiments 3 and 4, animals were either sham operated or adrenalectomized (ADX) prior to repeated stress, and blood samples were taken at the end of restraint on day 8 or day 5 to confirm ADX. All data are expressed as mean ± S.E.M. Asterisks (*) indicate repeated stress group values are significantly different than comparable acute restraint group values. Crosses (†) indicate ADX values are significantly different than comparable sham group values. 119

List of Abbreviations

AcH3	acetylated histone H3
ACTH	adrenocorticotrophic hormone
AR	adrenergic receptor
AVP	arginine vasopressin
BDNF	brain derived neurotrophic factor
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminals
cAMP	cyclic adenosine monophosphate
cDNA	circular deoxyribonucleic acid
CeA	central amygdala
CPM	counts per minute
CREB	c-AMP response element binding protein
CRF	corticotrophin-releasing factor
CS	conditioned stimulus
DNA	deoxyribonucleic acid
ERK	extracellular signal-regulated kinase
GABA	gamma-aminobutyric acid
GC	glucocorticoids
GR	glucocorticoid receptor
H3	histone H3
HPA	hypothalamic-pituitary-adrenal
LC	locus coeruleus
MeA	medial amygdala
MEK	MAPK/ERK kinase
mPFC	medial prefrontal cortex
mpPVN	medial parvocellular paraventricular nucleus of the hypothalamus
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NE	norepinephrine
pERK	phospho-extracellular signal-regulated kinase
PKA	protein kinase A
PVN	paraventricular nucleus of the hypothalamus
PVT	paraventricular nucleus of the thalamus
SAM	sympathetic-adrenal medullary
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
US	unconditioned stimulus

Abstract

Role of noradrenergic signaling in the basolateral amygdala in habituation to repeated stress

by

Nicola M. Grissom

Co-chairs: Theresa M. Lee and Seema Bhatnagar

The hypothalamic-pituitary-adrenal (HPA) axis is an important stress-responsive system, but overactivity of the HPA axis can be detrimental to the physiological and psychological health of the organism. HPA activity habituates with repeated exposure to a homotypic stressor to limit these negative health consequences. HPA activation to habituating stressors occurs via limbic brain structures, though the exact mechanisms are unknown. One limbic structure, the basolateral amygdala (BLA), is well known as a mediator of learning and memory for aversive events. In particular, noradrenergic signaling via the β-adrenergic receptor (β-AR) in the BLA can bidirectionally modulate memory for an aversive experience. I hypothesized that similar mechanisms in the BLA may regulate HPA habituation to a stressor. In this dissertation, I found that β-AR manipulations in the BLA after daily restraint bidirectionally modified the strength of habituation to restraint. β-AR blockade in the BLA prevented restraint-induced changes in gene expression in the hypothalamus and BLA. β-AR blockade also attenuated restraint-induced changes in intracellular signaling in the BLA, and these changes in

signaling exert direct control of HPA activity to restraint. These findings 1) demonstrate the importance of β−AR signaling in repeated restraint, 2) suggest that current models of β-AR function in the BLA need to be revised, and 3) suggest that habituation to repeated stressors has important similarities and differences to models of aversive conditioning.

Chapter 1.

Introduction

1.1 Overall introduction

Our environment presents us with stressors that we must overcome to survive and reproduce. Stressors can be defined broadly as a threat to the physical or psychological well-being of the organism (Armario, 2006; McEwen, 2008). Some stressors arrive in the form of a physical insult, such as hemorrhage or hypoxia, while others are psychologically distressing incidents, such as restraint or public speaking, and some are a mixture of the physical and the psychological. What is perhaps most interesting about our responses to these disparate kinds of threat is that physiologically speaking, they are largely the same (Armario, 2006; Dallman, 2007; Herman, Ostrander, Mueller, & Figueiredo, 2005; Sapolsky, Romero, & Munck, 2000; Sapolsky, 2003). Both activate physiological stress systems, including the hypothalamic-pituitary-adrenal (HPA) axis, with the purpose of enabling us to escape or challenge the stressor at hand. However, a key difference exists between predominantly physical and predominantly psychological stressors: repeated experience with psychological stressors can lead over time to a reduction of stress reactivity to the familiar (homotypic) stressor (Armario, 2006; Grissom & Bhatnagar, 2008). This phenomenon, termed habituation in the stress neurobiology literature, is a key mechanism for reducing the activation of metabolically costly stress systems to stimuli which prior experience has shown not to be truly threatening (Nesse, Bhatnagar, & Young, 2007).

How is this habituation accomplished? While predominantly physical stressors are able to directly activate the hypothalamus via brainstem nuclei to increase HPA activity, psychological stressors activate HPA activity via a distributed network of limbic brain regions (Herman & Cullinan, 1997; Herman, Ostrander, Mueller, & Figueiredo, 2005). Many of the same limbic regions have been established as regulators of emotional processing, learning, and memory (Davis, 2006; Lang & Davis, 2006; McGaugh, 2004;

Roozendaal, Hahn, Nathan, de Quervain, & McGaugh, 2004; Sandi & Pinelo-Nava, 2007; Sapolsky, 2003), perhaps indicating a common functionality linking stress responsivity to emotions and memory. The focus of this dissertation is to explore the role of one particular limbic region, the basolateral amygdala (BLA), in the development of habituation to repeated stressors. I will begin by discussing the HPA axis and habituation in further detail. Next, I will discuss the evidence that the BLA may be important to regulating HPA activity. As the BLA is most well-known for regulating emotional learning and memory, I will then discuss this literature in detail, focusing on the modulation of memory for aversive events by noradrenergic signaling in this region. Finally, I will integrate these literatures and propose my main hypothesis, that noradrenergic signaling in the BLA bidirectionally modulates habituation of HPA activity to a repeated stressor.

1.2 Stress responsive systems

In response to a stressor, a number of emergency systems are activated to aid in coping with the stressor. Stressful events can trigger behavioral escape responses (Bowers, Bilbo, Dhabhar, & Nelson, 2008; Chandramohan, Droste, Arthur, & Reul, 2008; Grissom, Kerr, & Bhatnagar, 2008; Maier, Ryan, Barksdale, & Kalin, 1986). All stressors activate a number of systemic physiological responses as well. One of these is the HPA axis, which will be described in further detail below. Another is the sympathetic-adrenal medullary (SAM) system, which stimulates the release of peripheral adrenaline to increase heart rate and blood perfusion of the brain and musculature (Sapolsky, Romero, & Munck, 2000). Yet another is the ascending noradrenergic system, in which brainstem structures, notably the locus coeruleus (LC), release norepinephrine (NE) throughout the CNS, focusing attention and enhancing memory consolidation (Aston-Jones, Chiang, & Alexinsky, 1991; Valentino & Van Bockstaele, 2008). Stressors rapidly and concurrently activate these multiple stress systems in response to an acute stressor (Armario, 2006; Sapolsky, Romero, & Munck, 2000; Valentino & Van Bockstaele, 2008). The HPA axis has been a particular focus of study of stress responses and adaptation in both rodents and humans, in part because of the profound effects of HPA hormones on tissues throughout the body, leading to physical and psychological pathologies that we shall see are

associated with disregulated HPA activity. After exposure to a stressor, HPA activation begins with signaling from stress-regulatory brain regions that ultimately activate the hypothalamus. A diagram of the key components of the HPA axis is shown in Figure 1.1. These signals drive the medial parvocellular neurons of the paraventricular nucleus of the hypothalamus (mpPVN) to release corticotrophin releasing factor (CRF) and other secretagogues, notably arginine vasopressin (AVP), into hypophyseal portal circulation around the pituitary. These secretagogues work in concert to activate the anterior pituitary to synthesize and cleave pro-opiomelanocortin into β–endorphin and adrenocorticotrophic hormone (ACTH). ACTH is released by the pituitary into general circulation and exerts its actions at the adrenal glands. At the adrenal cortices, ACTH binds to the melanocortin-2 receptor to activate cAMP signaling, where it stimulates the synthesis and release of glucocorticoids. In the rodent, the primary glucocorticoid is corticosterone, and in primates, it is cortisol. Glucocorticoids exert effects on a large number of tissues in the body, and are the primary functional endpoint of HPA activation (for further review of the HPA axis see Armario, 2006; Dallman & Jones, 1973; de Kloet, 2000).

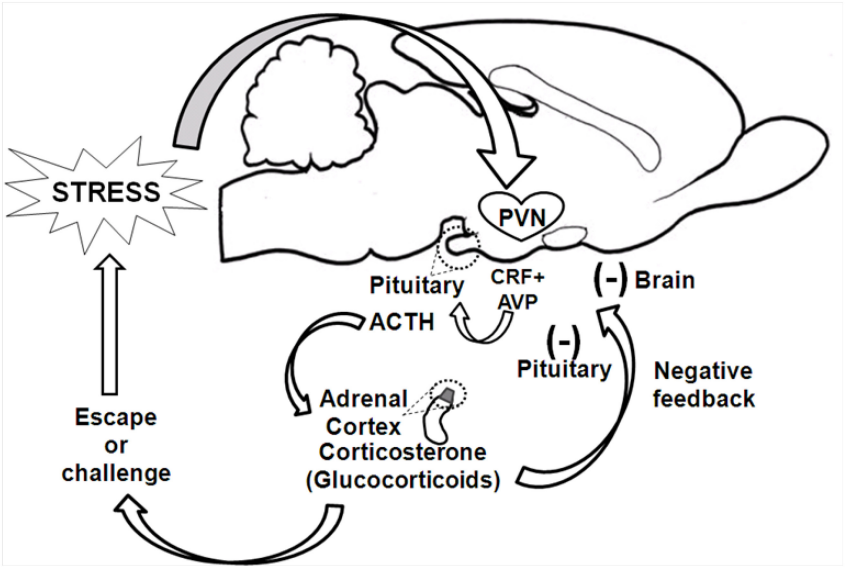

Figure 1.1 Schematic of the HPA axis. Stress activates the PVN (hypothalamus) to secrete the neuropeptides CRF and AVP. These peptides stimulate the anterior pitutiary to synthesize and release ACTH. ACTH travels through general circulation to the adrenal gland, located above the kidney, and stimulates the adrenal cortex to synthesize and release glucocorticoids (corticosterone in rodents). Glucocorticoids exert wide ranging physiological effects to enable the organism to escape or challenge the stressor at hand. Glucocorticoids also provide negative feedback at the level of the brain and pituitary to inhibit further HPA activity. See text for further details.

It should be noted that while the function of ACTH is to stimulate glucocorticoid release, for many reasons circulating glucocorticoid concentrations do not always parallel ACTH. First, ACTH stimulation of glucocorticoids necessarily lags hypothalamic stimulation of ACTH, due to the time necessary for ACTH to reach and activate the adrenal cortices. This means that peak elevation of ACTH may precede peak glucocorticoid elevation, and thus repeated sampling over a timecourse during and after stress is necessary to discern parallels in ACTH and glucocorticoid secretion. Second, the cAMP response in the adrenals to ACTH quickly plateaus, even as the ACTH concentration in the bloodstream continues to rise (Dallman & Jones, 1973; Dallman, Akana, Cascio, Darlington, Jacobson, & Levin, 1987), further limiting the glucocorticoid response relative to the ACTH response. Third, the waters have been muddied by the discovery that sympathetic splanchnic nerve stimulation to the adrenals, necessary for stimulating adrenaline release, also co-stimulates the release of glucocorticoids (Ehrhart-Bornstein & Bornstein, 2008; Ulrich-Lai, Arnhold, & Engeland, 2006). This splanchnic nerve-regulated release of glucocorticoids is independent of ACTH release, so although the activities of the stress-responsive systems parallel one another, sympathetic inputs to the adrenal mitigate the ACTH-specific effects on the adrenals. Fourth, under conditions of repeated stress the adrenals can change in sensitivity to ACTH (Armario, Restrepo, Castellanos, & Balasch, 1985; Armario, Hidalgo, & Giralt, 1988). For these reasons, investigations into the neural regulation of HPA activity in animals sometimes see dissociations between relative ACTH and glucocorticoid levels. It is believed that ACTH is more reflective of the true psychological state or level of stress (Armario, 2006) as there are fewer intervening factors between the brain and pituitary that mitigate the effects of stressors on ACTH release than there are between the brain and adrenals that mitigate the effects of stressors on glucocorticoid release.

Glucocorticoids are the key mediator of HPA effects on physiology and behavior, as well as crucial to providing negative feedback to terminate stress-induced HPA activity. These effects are mediated by the two kinds of glucocorticoid receptor, the high-affinity mineralocorticoid receptor (MR) and the lower-affinity glucocorticoid receptor (GR). As glucocorticoids are lipophilic, MR and GR are localized within the cell and when bound act primarily as transcription factors, mediating the long-term effects (hours +) of

glucocorticoids (Dallman, Akana, Cascio, Darlington, Jacobson, & Levin, 1987; Sapolsky, Romero, & Munck, 2000). However, there are rapid actions of glucocorticoids as well, thought to be mediated by a putative membrane-bound receptor (de Kloet, 2000; Sapolsky, Romero, & Munck, 2000). The high-affinity MR are largely occupied by basally circulating concentrations of glucocorticoids, so stress-levels of glucocorticoids are thought to act primarily via GR (de Kloet, 2000; Lightman, 2008).

The regulation of glucocorticoid release is of profound psychological and biological significance. Glucocorticoids exert wide-ranging physiological effects, activating some systems that may confer survival benefit to the organism, such as increasing catabolic processes to provide increased energy to the organism and enhancing memory. Simultaneously, glucocorticoids suppress nonessential physiological functions such as immune function and gonadal hormone secretion, in order to divert metabolic resources to escaping or challenging the stressor at hand (Dallman, 2007; Sapolsky, Romero, & Munck, 2000). These actions can be advantageous when activated in response to an unknown or truly harmful stressor, but it is not difficult to see that hypoactive HPA responses to a true threat may prevent survival, while hyperactive HPA responses in response to nonharmful situations can over time result in deleterious outcomes. Thus, disregulated HPA activity is associated with a host of dysfunctions including immune suppression, cardiac morbidity, metabolic disorder, impaired learning, memory, and cognition, hippocampal neuronal atrophy, major depression and post-traumatic stress disorder (Golier, Schmeidler, Legge, & Yehuda, 2007; Sapolsky, Romero, & Munck, 2000; Simeon, Knutelska, Yehuda, Putnam, Schmeidler, & Smith, 2007; Thomson & Craighead, 2007; Yehuda, Teicher, Trestman, Levengood, & Siever, 1996). Some of these outcomes, such as immune suppression and hippocampal neuronal atrophy, are directly affected by glucocorticoids, whereas for other outcomes the link between HPA disregulation and morbidity is unknown.

Stress-induced elevations in HPA activity are therefore ideally limited to situations where it is most necessary to survival. Most stressors are experienced on a backdrop of previous stressful experiences, and it is noteworthy that HPA activity to today's stressor is dependent on yesterday's stressor. When a human or rat experiences the same stressor repeatedly (homotypic stress), even in the context of other, novel stressors (Armario,

2006); (Simpkiss & Devine, 2003), they begin to show an adaptation of response to the increasingly familiar homotypic stressor. This is seen as a reduction of ACTH and corticosterone responses elicited by the stressor itself and/or a more rapid return to baseline following termination of the stressor. This experience-dependent change in stress reactivity to a homotypic stressor is termed "habituation" in the stress neurobiology literature. The ability to habituate HPA responses to a familiar stressor is thought to have strong evolutionary utility as it conserves energy and resources by dampening responses to stressors that experience has shown are not life-threatening (Nesse, Bhatnagar, & Young, 2007). It should be noted that habituation to a repeated homotypic stressor not limited to HPA activity, but reflects a broad-spectrum reduction in responses elicited by stressors including behavioral avoidance or escape (Grissom, Kerr, & Bhatnagar, 2008; Ruys, Mendoza, Capitanio, & Mason, 2004; Weinberg, Bhatt, Girotti, Masini, Day, Campeau, & Spencer, 2008, see Appendix A), and SAM activation as measured in cardiovascular output (Costoli, Bartolomucci, Graiani, Stilli, Laviola, & Sgoifo, 2004; Dobrakovova, Kvetnansky, Oprsalova, & Jezova, 1993; Kvetnansky, Pacak, Fukuhara, Viskupic, Hiremagalur, Nankova, Goldstein, Sabban, & Kopin, 1995; Sapolsky, Romero, & Munck, 2000; Schommer, Hellhammer, & Kirschbaum, 2003).

The evolutionary significance of habituating to previously experienced, not ultimately harmful stressors is underscored by the fact that it has been noted in a number of species (though most frequently studied in laboratory rodents and humans) and a wide number of stressors. However, it should be noted that stressors that lead to habituation are characterized by having psychological aspects, which repeated experience shows are not inherently harmful (Herman, Ostrander, Mueller, & Figueiredo, 2005). For example, in animals habituation of HPA activity has been seen to such diverse stressors as restraint (Bhatnagar, Huber, Nowak, & Trotter, 2002; Girotti, Weinberg, & Spencer, 2007; Gomez, Houshyar, & Dallman, 2002; Jaferi, Nowak, & Bhatnagar, 2003; Jaferi & Bhatnagar, 2006; Jaferi & Bhatnagar, 2007; Keim & Sigg, 1976; Lunga & Herbert, 2004; Ma, Lightman, & Aguilera, 1999; McQuade, Tamashiro, Wood, Herman, McEwen, Sakai, Zhang, & Xu, 2006; Natelson, Ottenweller, Cook, Pitman, McCarty, & Tapp, 1988; Simpkiss & Devine, 2003; Weinberg, Bhatt, Girotti, Masini, Day, Campeau, & Spencer, 2008), intermittent exposure to cold (Bhatnagar & Meaney, 1995; Bhatnagar,

Mitchell, Betito, Boksa, & Meaney, 1995; Kant, Bunnell, Mougey, Pennington, & Meyerhoff, 1983), novel environment (Bassett, Cairncross, & King, 1973; Johnson & Moberg, 1980; Muir & Pfister, 1987; Pfister, 1979), immobilization (Garcia, Marti, Valles, Dal-Zotto, & Armario, 2000; Giralt, Garcia-Marquez, & Armario, 1987; Hauger, Lorang, Irwin, & Aguilera, 1990), water immersion without swimming (De Boer, Koopmans, Slangen, & Van der Gugten, 1990), noise (Armario, Castellanos, & Balasch, 1984; Armario, Lopez-Calderon, Jolin, & Balasch, 1986; Borrell, Torrellas, Guaza, & Borrell, 1980; De Boer, Van der Gugten, & Slangen, 1989), handling (Dobrakovova & Jurcovicova, 1984; Dobrakovova, Kvetnansky, Oprsalova, & Jezova, 1993), and repeated ethanol injection (Spencer & McEwen, 1990). Habituation of HPA activity in humans has been demonstrated to repeated psychosocial stressors such as the Trier Social Stress Test (Gerra, Zaimovic, Mascetti, Gardini, Zambelli, Timpano, Raggi, & Brambilla, 2001; Gunnar, Connors, & Isensee, 1989; Kirschbaum, Prussner, Stone, Federenko, Gaab, Lintz, Schommer, & Hellhammer, 1995; Schommer, Hellhammer, & Kirschbaum, 2003; Wust, Federenko, van Rossum, Koper, & Hellhammer, 2005) and to repeated parachute jumps (Deinzer, Kirschbaum, Gresele, & Hellhammer, 1997).

Given the significance of experience-dependent changes in HPA activity via habituation, it is no surprise that its regulation has been a focus for investigation for decades. Habituation to repeated stressors is a two-part, iterative process: it is acquired or develops with each stress experience (*n)*, and it is expressed during the following homotypic stress experience (*n+1*). The *n* of stress experiences necessary to see a significant reduction in stress responses differs between stressors, and between dependent measures to the same stressor. For instance, habituation is more rapid to the milder stressor of restraint than to immobilization, which is more severe (Garcia, Marti, Valles, Dal-Zotto, & Armario, 2000; Giralt, Garcia-Marquez, & Armario, 1987; Hauger, Lorang, Irwin, & Aguilera, 1990; Ma & Lightman, 1998; Vogel & Jensh, 1988). Habituation of c-fos in limbic brain regions in response to the stressor of daily predator odor precedes the habituation of HPA activity by several days (Weinberg, Bhatt, Girotti, Masini, Day, Campeau, & Spencer, 2008). These discrepancies in the rate of habituation may be due to the fact that responses to stress *n+1* are regulated by processes that occur during both the acquisition of habituation (*n*) and the expression of habituation (*n+1*).

There is good evidence to support a role for glucocorticoid negative feedback via MR and GR in mediating both the acquisition and expression of habituation to repeated stressors. Removal of the adrenal glands (adrenalectomy) results in a loss of circulating glucocorticoids. This technique is used alone or in combination with corticosterone replacement in animals to test the effect of removing stress-induced variations in corticosterone on habituation, or the role of low (average unstressed) versus high (average stressed) concentrations of corticosterone on habituation. In one such study, adrenalectomy in conjunction with high corticosterone replacement accelerated the habituation of hypothermic responses to repeated restraint in comparison to intact and low corticosterone replacement animals (Stamp & Herbert, 2001). This suggests that the enhanced negative feedback provided by chronic elevations in corticosterone is able to enhance habituation. However, this kind of study cannot address whether negative feedback plays an important role in acquisition or expression. The expression of habituation to repeated restraint was blocked by systemic blockade of MR (Cole, Kalman, Pace, Topczewski, Lowrey, & Spencer, 2000). To look at the development or acquisition of habituation, our lab has focused on one limbic brain region, the paraventricular thalamus (PVT), which when lesioned prevents habituation (Bhatnagar, Huber, Nowak, & Trotter, 2002). Chronic corticosterone implants in the PVT of adrenalectomized animals enhanced habituation (Jaferi & Bhatnagar, 2006), confirming that the negative feedback in this region was functionally relevant. Importantly, blocking MR and GR prior to daily restraint on days 1-7, during the acquisition or development of habituation, blocked habituation, but blocking these receptors prior to the test restraint on day 8 had no effect on the expression of habituation (Jaferi & Bhatnagar, 2006). Thus, while glucocorticoids play an important role in both the expression and acquisition of habituation, the role of corticosterone in the PVT is to regulate acquisition, not expression.

However, glucocorticoid negative feedback effects cannot fully explain experience-dependent changes in HPA activity. Habituation still occurs in adrenalectomized animals, though it may be enhanced by glucocorticoids (Jaferi & Bhatnagar, 2006; Stamp & Herbert, 2001). Thus, experience-dependent changes in stress responses are likely regulated by interactions between stressor-activated brain regions in addition to the

contributions of glucocorticoids. It is noteworthy that habituation of HPA activity to repeated stressors is a phenomenon limited to predominantly psychological stressors, as noted above (Herman, Ostrander, Mueller, & Figueiredo, 2005). Predominantly physical stressors are able to more directly activate the hypothalamus via brainstem nuclei without significantly involving higher brain structures, while stressors that are predominantly psychological activate a host of limbic brain regions in association with the activation of the hypothalamus (Herman, Ostrander, Mueller, & Figueiredo, 2005). This evidence indicates that these limbic brain structures may be of particular importance in regulating habituation. In the next section, I will discuss the evidence implicating one such limbic structure, the BLA, in regulating experience-dependent changes in stress reactivity.

1.3 Neural regulation of adaptive responses to repeated stress

A significant number of limbic brain regions are of interest as potential regulators of HPA activity, including the basolateral, medial, and central amygdalar nuclei, the hippocampus, the prefrontal cortex, and the paraventricular thalamus (Carter, Pinnock, & Herbert, 2004; Herman, Figueiredo, Mueller, Ulrich-Lai, Ostrander, Choi, & Cullinan, 2003; Jaferi & Bhatnagar, 2006; Pacak & Palkovits, 2001; Pardon, Ma, & Morilak, 2003). There are specific findings that suggest that the BLA deserves special focus in this field. Much of this evidence indicates that the BLA is preferentially activated by under conditions of repeated stressor exposure. The BLA exhibits a specific increase in Fos activation in animals exposed to novel restraint following repeated cold exposure as compared to restrained animals with no prior exposure to the cold stressor (Bhatnagar & Dallman, 1998). Chronic stressors induce hippocampal neuronal atrophy, a process that has been implicated in the pathophysiology of post-traumatic stress disorder (Sapolsky, 2000; Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002; Yehuda & LeDoux, 2007). Interestingly, however, a protocol of chronic immobilization that leads to hippocampal atrophy leads to simultaneous BLA neuronal hypertrophy (Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002; Vyas, Pillai, & Chattarji, 2004). BLA hypertrophy in these animals is associated with increased anxiety (Mitra, Vyas, Chatterjee, & Chattarji, 2005; Vyas, Pillai, & Chattarji, 2004). Furthermore, this BLA hypertrophy is maintained three weeks after the termination of immobilization, at which

point hippocampal atrophy has recovered (Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002; Vyas, Pillai, & Chattarji, 2004). Our laboratory has found that habituation to repeated restraint persists three weeks after the termination of restraint (Bhatnagar, Huber, Nowak, & Trotter, 2002), indicating that habituation induced a long-term change in neuronal function. Anatomically, the BLA is well positioned to communicate with all the limbic regions implicated in psychological stress responses, including the mPFC, hippocampus, paraventricular thalamus, central amygdala, and bed nucleus of the stria terminalis (BNST), and most directly impinge on the PVN via the BNST (Davis, 2006; Ishikawa & Nakamura, 2003; Ottersen, 1982; Sullivan, Apergis, Bush, Johnson, Hou, & Ledoux, 2004).

However, the current literature examining the effect of BLA manipulations on the habituation of HPA activity is limited and has not led to easy interpretation. Lesions of the entire amygdala, including the BLA, central amygdala (CeA), and medial amygdala (MeA), delayed but did not prevent the habituation to a repeated stressor as measured by c-fos activation at the PVN (Carter, Pinnock, & Herbert, 2004). This delay of habituation was not due to the CeA lesion, as specific lesions of this structure had no effect on the timecourse of habituation. In contrast, specific lesions of the BLA were found to reduce HPA activity in both acutely stressed and repeatedly stressed animals to concentrations comparable with an intact, habituated animal (Bhatnagar, Vining, & Denski, 2004). Finally, there is a limited role for the BLA in the expression of HPA habituation. Animals were given repeated restraint, repeated cold exposure, or no treatment for seven days. All groups were restrained on the 8th day, and immediately prior to restraint muscimol (a GABA-a receptor agonist) was administered to the BLA. Intra-BLA GABA activation enhanced HPA activity in the animals receiving novel restraint after prior cold, but did not affect the expression of HPA habituation in the repeatedly restrained animals (Bhatnagar, Vining, & Denski, 2004). Collectively, this literature does not indicate a clear role for the BLA in responses to stress, but it is notable that the role of the BLA in the acquisition of habituation to repeated stressors has not been investigated.

1.4 The BLA in aversive learning

In fact, the BLA plays a particularly important role in the acquisition of aversive learning, including Pavlovian fear conditioning and inhibitory avoidance paradigms (Bonini, Cammarota, Kerr, Bevilaqua, & Izquierdo, 2005; Lang & Davis, 2006; Maren, 2003; McGaugh, 2004; Sandi, Cordero, Ugolini, Varea, Caberlotto, & Large, 2008). In the case of Pavlovian fear conditioning, the BLA is a crucial site of plasticity for forming Pavlovian associations between conditioned stimuli (CS) such as a tone, and the unconditioned stimulus (US) of footshock, permitting learned freezing to tone (Maren, 2003; Rabinak & Maren, 2008; Zimmerman, Rabinak, McLachlan, & Maren, 2007). Importantly to our discussion, lesions of the BLA profoundly limit the ability of the animal to form a CS-US association, and only with extensive overtraining (75 shock-tone pairings) can animals with BLA lesions acquire freezing to tone (Zimmerman, Rabinak, McLachlan, & Maren, 2007). It would not appear that lesions of the BLA in those studies examining HPA activity caused such a profound deficit in the ability of the animals to acquire and/or express habituation. Thus if the BLA plays a role in acquisition of experience-dependent changes in HPA activity elicited by homotypic stressors, it is probably not mediated by Pavlovian associations occurring within the BLA.

Inhibitory avoidance paradigms are fundamentally operant rather than Pavlovian in nature (Maren, 2003; Rossato, Bonini, Coitinho, Vianna, Medina, Cammarota, & Izquierdo, 2004). In these, an animal learns an avoidance response to an aversive stimulus, such as a shock probe. The degree of learning and memory for the training is measured by the latency to contact the probe. The BLA has been found to exert a substantial modulatory influence on the acquisition of inhibitory avoidance conditioning. Over several decades, a large body of research has implicated ascending noradrenergic (NE) signaling in the BLA in both blocking and enhancing the consolidation of memory for inhibitory avoidance training (McGaugh, 2004; McIntyre, Power, Roozendaal, & McGaugh, 2003). In these experiments, animals trained normally, after which the BLA manipulations were performed, and recall of training was tested under drug-free conditions. Thus, the effect of BLA manipulation was to alter consolidation processes important to long-term acquisition, without directly interfering with training or the expression of the avoidance response. In these experiments, the effect of NE in the BLA

has been shown to be due to activation of the β−adrenergic receptor (β−AR), and results in an inverted-U dose-response curve (Ferry & McGaugh, 1999; Ferry, Roozendaal, & McGaugh, 1999; Roozendaal, Quirarte, & McGaugh, 2002). Very low or very high levels of NE in the BLA at the end of training are detrimental to memory consolidation, and lead to decreased avoidance of the shock-probe, compared to intermediate doses of NE (McIntyre, Hatfield, & McGaugh, 2002; McIntyre, Miyashita, Setlow, Marjon, Steward, Guzowski, & McGaugh, 2005). β−AR agonists infused in the BLA after training similarly enhance memory consolidation (Ferry & McGaugh, 1999; Roozendaal, Quirarte, & McGaugh, 2002; Roozendaal, Schelling, & McGaugh, 2008). β−AR antagonists infused in the BLA, or given systemically with intra-BLA NE, block the consolidation-enhancing effects of intermediate NE (Ferry, Roozendaal, & McGaugh, 1999; Roozendaal, Hahn, Nathan, de Quervain, & McGaugh, 2004; Roozendaal, Okuda, Van der Zee, & McGaugh, 2006; Roozendaal, Schelling, & McGaugh, 2008). Notably, recent work has established that other stress-response-regulatory compounds, such as CRF and corticosterone, acting in the BLA also enhance memory consolidation, but the effects of these compounds appears to be secondary to β−AR activation (Roozendaal, Brunson, Holloway, McGaugh, & Baram, 2002; Roozendaal, Quirarte, & McGaugh, 2002; Roozendaal, Griffith, Buranday, de Quervain, & McGaugh, 2003; Roozendaal, Hahn, Nathan, de Quervain, & McGaugh, 2004; Roozendaal, Okuda, de Quervain, & McGaugh, 2006; Roozendaal, Schelling, & McGaugh, 2008).

Intra-BLA β−AR modulation has since been shown to affect the consolidation of training-related memory for tasks other than inhibitory avoidance, such as the Morris water maze (Hatfield & McGaugh, 1999) and conditioned taste aversion (Miranda, Rodri Guez-Garci, Reyes-Lopez, Ferry, & Ferreira, 2008). The original consolidation of Pavlovian fear conditioning seems to rely on BLA plasticity occurring only during the training (Wilensky, Schafe, & Ledoux, 2000), but BLA β-AR manipulations after re-exposure to the training context affect the reconsolidation of Pavlovian fear conditioning (Debiec & Ledoux, 2004) and manipulation of PKA, which is downstream of β-AR, in the BLA also affects reconsolidation (Tronson, Wiseman, Olausson, & Taylor, 2006). The bidirectionality of β−AR effects in the BLA, and the wide variety of paradigms

which this manipulation affects, suggests a broad function for the BLA, in addition to its role in Pavlovian CS-US associations. It may modulate processes occurring after an initial experience to augment or diminish the acquisition of experience-dependent change in subsequent behavior.

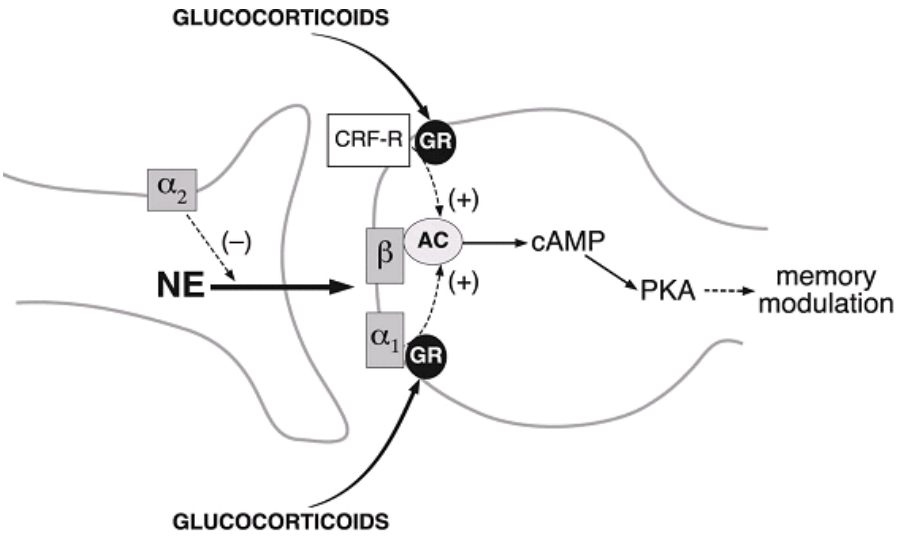

Figure 1.2 Summary of McGaugh model of β-AR function in the BLA. Taken from Roozendaal, Schelling, and McGaugh, 2008. In this model β-AR constitute the primary mechanism by which NE modulates memory consolidation. The actions of glucocorticoids, acting via GR, of CRF acting through the CRF-1 receptor, and of α1-AR stimulation is to enhance the actions of β-AR signaling to PKA. α2-AR are depicted as solely presynaptic autoreceptors which inhibit NE release into the synaptic cleft. PKA activation via cAMP is thought to underlie the modulation of memory consolidation, though the exact mechanisms are unclear.

Returning with this insight to the HPA literature, it is noteworthy that NE signaling in a number of brain regions other than the BLA has been shown to alter HPA activity. NE facilitates HPA activity when applied to the PVN, mPFC, lateral septum, BNST, MeA, and CeA (Pacak, Palkovits, Kopin, & Goldstein, 1995; Pardon, Gould, Garcia, Phillips, Cook, Miller, Mason, & Morilak, 2002; Pardon, Ma, & Morilak, 2003). Strain differences in NE signaling are inversely associated with strain differences in acute HPA regulation (Pardon et al., 2002). Blockade of NE actions at one region, the BNST, attenuates acute HPA responses (Pardon et al., 2003). Thus, there is every reason to believe that NE signaling is being increased by stress in the BLA as it is in these many other structures, and that NE signaling is relevant to HPA activity. Within the BLA, NE has been found to inhibit BLA projection neurons (Buffalari & Grace, 2007) and repeated stress leads to a

disinhibition of these neurons by NE (Buffalari & Grace, 2009). Thus, the effects of NE signaling in the BLA are sensitive to the animal's prior stressor exposure. Collectively, these findings suggest that the effects of NE signaling in the BLA may be functionally relevant to HPA activity.

To summarize, the BLA may be involved in the acquisition of habituated responses to repeated stressors, although it is not required. The BLA has already been demonstrated to be of importance to the acquisition of avoidance responses. This is accomplished by the modulation of consolidation of memory for training. Bidirectional modulation of memory consolidation is mediated in the BLA by NE signaling to β−AR. NE signaling has been found to exert profound effects on HPA activity in a variety of stress-regulatory brain regions. Together, these observations lead to the hypothesis that intra-BLA NE signaling via β−AR may play a role in the acquisition of habituated responses to repeated stressors. This dissertation tests this hypothesis.

1.5 Overall hypothesis and specific aims

The overall goal of this dissertation is to examine the role of the BLA in experience-dependent changes in stress responses. Among the most significant of the experience-dependent changes induced by repeated homotypic stressors is the habituation of HPA activity. Repeated restraint is one of the most frequently used habituating stressors in rodents, and so I examined HPA responses to this stressor induced by prior restraint exposure and intra-BLA manipulations of NE signaling.

I first tested the overall hypothesis that manipulating β−AR receptors in the BLA after daily restraint exposure could bidirectionally affect the acquisition of habituation, modifying subsequent HPA activity to restraint. In these experiments, I also examined the effect of β-AR blockade on the acquisition of habituated struggling behavior to restraint, to establish that multiple modalities of habituation were altered by my manipulation. Finding support for intra-BLA β−AR modulation of habituation, I next examined changes in gene expression in stress-regulatory brain regions to determine what neuronal differences that accompany habituation are altered by intra BLA β−AR manipulation. Finally, I examined changes in intracellular signaling within the BLA that might account

for the effects of β−AR manipulation on habituation. I then directly manipulated intracellular signaling to determine if the changes seen in the prior experiment were functionally relevant to HPA activity.

Specific Aim 1 (Chapter 2) To determine the effects of β−AR antagonist or agonist in the BLA after daily restraint on the acquisition of habituation to repeated restraint.

Justification and approach: The role of the BLA in regulating stress responses, including HPA activity, may be as a modulator of experience-dependent changes, and this modulation may be subserved by NE β−AR signaling in the BLA. I tested the hypothesis that changing the activity of β−AR in the BLA can modulate the acquisition of habituated responses to repeated restraint. I specifically predicted that infusing a β−AR antagonist into the BLA after daily restraint would prevent subsequent habituation to restraint, whereas infusing a β−AR agonist under similar conditions would enhance habituation to restraint. I observed that intra-BLA infusion of a β−AR antagonist immediately after daily restraint prevented the habituation of ACTH activity and struggling responses to a test restraint. Daily intra-BLA β−AR blockade 4 hours post-restraint did not affect habituation, indicating that β−AR effects were limited to the immediate post-stressor period. In contrast, a low dose of intra BLA β−AR agonist was found to enhance the habituation of ACTH activity to restraint.

Specific Aim 2 (Chapter 3) To determine if changes in peptide and neurotrophin gene expression in stress-related brain structures induced by repeated restraint are prevented by β−AR antagonist following daily restraint.

Justification and approach: Having found in Chapter 2 that infusion of a β−AR antagonist into the BLA after daily restraint prevented habituation of HPA activity and behavior to restraint, I wanted to determine to what degree this manipulation would affect the limbic network involved in regulating stress responses. I hypothesized that intra-BLA β−AR blockade would prevent repeated stress-induced changes in mRNA expression in several different brain regions. I elected to begin by examining changes in stress peptide mRNA expression, specifically CRF and AVP in the PVN. AVP in this region is upregulated by repeated stress (Ma & Lightman, 1998). I also examined changes in neurotrophin mRNA

expression in the BLA and hippocampus that have previously been shown to be downregulated by repeated stress (Tsankova, Berton, Renthal, Kumar, Neve, & Nestler, 2006). Overall, I found that repeated restraint increased AVP mRNA in the PVN, and as hypothesized, this increase was blocked by post-restraint intra-BLA β−AR blockade. Repeated restraint also increased BDNF mRNA in the BLA, and this change was blocked by the BLA manipulation.

Specific Aim 3 (Chapter 4) To determine the changes in intracellular signaling in the BLA modified by repeated restraint and β−AR antagonism

Justification and approach: There is a limited amount of research about the specific effect of β−AR stimulation in the BLA that could account for how blocking this receptor in this region can subsequently influence functioning in other brain regions. Some recent work suggests that the effects of β−AR could be mediated by intracellular ERK signaling. β−AR are already known to activate PKA signaling, and both PKA and ERK are known to regulate mechanisms that alter epigenetic marks such as histone acetylation, thereby enhancing the availability of DNA for transcription. I first examined the BLA of animals at the end of 30 minute restraint and discovered that compared to unrestrained animals, restrained animals exhibited decreased ERK activation, and increased histone acetylation. This led me to examine the effects of repeated restraint and post-stress β−AR blockade in animals sacrificed basally. In these animals, repeated restraint led to an overall decrease in ERK activation and an overall increase in histone acetylation. These repeated restraint induced changes were not seen in repeatedly restrained animals given intra-BLA blockade. Finally, I pharmacologically blocked ERK activation in the BLA and found that this decreased HPA activity in all groups, regardless of prior restraint experience.

Overall, these experiments make a strong case for the involvement of the BLA in habituation of HPA activity and struggling behavior to repeated stress. This is subserved by NE signaling at β-AR, which when blocked in the BLA after daily stress, prevents HPA and behavioral habituation, changes in the hypothalamus and the BLA associated with habituation, including a decrease in pERK. Pharmacologically decreasing pERK in the BLA leads to a reduction in HPA activity to restraint even in restraint-naïve animals. These results suggest that the BLA may be modulating processes occurring in the BLA

and/or elsewhere in the brain that allow for the changes in HPA activity to occur. In the final chapter, I discuss the significance of this work to models of NE action in the BLA and our understanding of the physiological and psychological mechanisms that underlie habituation to repeated stress.

Chapter 2.

β−AR signaling in the BLA bidirectionally modifies the habituation of HPA and behavioral responses to repeated restraint

2.1 Introduction

Upon first exposure to a stressful event, the hypothalamic-pituitary-adrenal (HPA) axis is activated, enabling the organism to fight or flee via the actions of glucocorticoid release. However, as an organism becomes increasingly familiar with a particular stressor, the HPA activation elicited by that stressor can be reduced or habituated (Armario, 2006; Girotti, Pace, Gaylord, Rubin, Herman, & Spencer, 2006; Grissom & Bhatnagar, 2008; Herman, Ostrander, Mueller, & Figueiredo, 2005; Weinberg, Bhatt, Girotti, Masini, Day, Campeau, & Spencer, 2008). Habituation of HPA activity to repeated stressors is an adaptive change that limits the risk of physiological and psychological dysfunction associated with elevated glucocorticoid release (McEwen, 2008; Nesse, Bhatnagar, & Young, 2007; Sapolsky, Romero, & Munck, 2000).

The basolateral amygdala (BLA) is a region of interest in habituation because repeated stressors induce alterations in its structure and function. Daily intermittent exposure to a cold environment leads to increased Fos protein in a limited number of regions including the BLA, but not such regions as the medial amygdala, medial prefrontal cortex, or BNST, in response to a novel restraint as compared to a naïve animal undergoing novel restraint (Bhatnagar & Dallman, 1998). Ten days of immobilization for 2 hours a day induces dendritic hypertrophy in the BLA that persists for at least 3 weeks following stress termination (Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002; Vyas, Pillai, & Chattarji, 2004; Vyas, Jadhav, & Chattarji, 2006). This BLA dendritic hypertrophy occurs even as the hippocampus undergoes dendritic atrophy (Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002; Vyas, Pillai, & Chattarji, 2004). This evidence suggests that the BLA is sensitive to the fact that the animal has undergone prior repeated stress.

The BLA is also well known as a region regulating the acquisition and expression of emotional learning and memory (Maren, 2005; McGaugh, 2004; Sandi & Pinelo-Nava, 2007), and it has led to the hypothesis that memory mechanisms in the BLA may regulate responses to previously experienced stressors (Carter, Pinnock, & Herbert, 2004). In particular, the BLA is a crucial locus for the acquisition of a wide variety of aversive tasks, including inhibitory avoidance, the Morris water maze, and conditioned taste aversion (Hatfield & McGaugh, 1999; Huff, Frank, Wright-Hardesty, Sprunger, Matus-Amat, Higgins, & Rudy, 2006; Miranda, Rodri Guez-Garci, Reyes-Lopez, Ferry, & Ferreira, 2008; Roozendaal, Griffith, Buranday, de Quervain, & McGaugh, 2003; Roozendaal, Okuda, de Quervain, & McGaugh, 2006; Roozendaal, Schelling, & McGaugh, 2008). In these paradigms, the BLA modulates the consolidation of long-term memory occurring after training. This modulation is accomplished via NE signaling at β-AR in the BLA. After training on a task, the activation state of β-AR within the BLA immediately after training profoundly influences the consolidation of memory for the task. Post-training administration of NE or β-AR agonists enhance the consolidation or reconsolidation of training-associated memories (Ferry & McGaugh, 1999; McIntyre, Hatfield, & McGaugh, 2002; McIntyre, Miyashita, Setlow, Marjon, Steward, Guzowski, & McGaugh, 2005). Administration of β-AR antagonists after training disrupts these processes (Debiec & Ledoux, 2004; Ferry, Roozendaal, & McGaugh, 1999; Roozendaal, Brunson, Holloway, McGaugh, & Baram, 2002; Roozendaal, Okuda, Van der Zee, & McGaugh, 2006). These bidirectional effects suggest a broad role for NE signaling the BLA in modulating the acquisition of experience-dependent changes in response (McGaugh, 2004; McGaugh & Roozendaal, 2008). As reductions in HPA response to a familiar stressor is dependent on prior experience with the stressor, it is possible that NE signaling in the BLA may regulated habituation to repeated stress as one form of experience-dependent change in response.

NE signaling in stress-response-regulatory brain regions other than the BLA alters HPA activity and other responses to stressors (Cecchi, Khoshbouei, & Morilak, 2002; Ma & Morilak, 2005; Pardon, Gould, Garcia, Phillips, Cook, Miller, Mason, & Morilak, 2002; Pardon, Ma, & Morilak, 2003). Blocking the actions of NE on α1-AR in the CeA

prevents the acute behavioral effects of a single immobilization (Cecchi, Khoshbouei, & Morilak, 2002). Blocking α1-AR in the PVN or BNST reduces HPA activity to novel stress in previously stressed animals (Ma & Morilak, 2005; Pardon, Ma, & Morilak, 2003). β-AR effects on acute HPA activity in these experiments were only studied in the CeA, which contains no β-AR (Rainbow, Parsons, & Wolfe, 1984). Morilak and colleagues have also found strain differences in HPA activity to stress related to NE signaling. Compared to the Sprague-Dawley rat strain, NE signaling in the BNST is diminished in the depressive-like Wistar-Kyoto rat, and this is associated with hyperactive ACTH activity to a single immobilization (Pardon, Gould, Garcia, Phillips, Cook, Miller, Mason, & Morilak, 2002). This research has demonstrated the importance of NE signaling in limbic regions other than the BLA in regulating stress responses.

The sum of this evidence indicates: 1) among the limbic regions involved in stress responses, the BLA is sensitive to prior stress history; 2) NE signaling in the BLA via β-AR bidirectionally regulates the consolidation of memories for aversive experiences; 3) NE signaling in other limbic regions regulates the degree of HPA activity elicited by a stress experience. I hypothesize that the BLA may play a role in permitting the acquisition of HPA habituation via NE activation of β-AR. The level of β-AR activation in the BLA after daily repeated stress ("training") may modulate the acquisition of habituation to the repeated stressor. I tested this hypothesis in three experiments. First, I infused β-AR antagonist into the BLA after daily repeated restraint to determine if this would prevent habituation of HPA activity to a test restraint when no drug was administered. As part of this first experiment, I tested whether this manipulation was effective when not delivered immediately after training, as there is a window for the effects of β-AR and related manipulations in the BLA on memory consolidation (McGaugh, 2004; Tronson, Wiseman, Olausson, & Taylor, 2006). Second, I tested whether β-AR blockade after daily restraint would prevent behavioral habituation to a test restraint as it prevented HPA habituation. We have recently demonstrated that in the case of repeated restraint stress, animals habituate behaviorally as well as hormonally, by reducing the time spent struggling during the inescapable situation (Grissom, Kerr, & Bhatnagar, 2008, Appendix A). This would demonstrate that the role of BLA β-AR

signaling in habituation to a repeated stressor is not limited to HPA activity, but reflects multiple modalities of adaptation to a familiar stressor. Finally, I administered a β-AR agonist after daily restraint to test whether this would enhance habituation of HPA activity to a test restraint. In sum, these studies will test whether β-AR manipulation in the BLA after repeated restraint can enhance or diminish HPA and behavioral adaptation to repeated restraint.

2.2 Methods

Animals

Male Sprague-Dawley rats (Charles Rivers, MA) weighing between 225–250 g were individually housed in plastic tub cages with ad libitum access to food and water. The housing room was on a 12:12 L:D cycle with lights on at 0600h. Animals were given a 5–7 day acclimation period prior to the beginning of experimentation or surgery and were briefly handled during this period. All stress and experimentation took place between 0800 – 1200h. All procedures were approved by the UCUCA at the University of Michigan and/or the IACUC at the Children's Hospital of Philadelphia.

Experimental Design

A layout of the design of Experiment 2.1 is shown in Figure 2.1. The designs of subsequent experiments in this dissertation are adapted from this design. Crucially, in all experiments, drug administration occurred only after daily restraint, and no drugs were administered on day 5, when HPA or behavioral measurements were taken. Thus, all differences in response on day 5 are due to prior restraint and intra-BLA drug administration on days 1-4.

Experiment 2.1: What is the effect of intra-BLA β-AR blockade during repeated restraint on HPA habituation to restraint?

This study tested the hypothesis that intra-BLA β-AR antagonist administered after daily exposure to restraint would prevent the development of habituated HPA responses to repeated restraint. In addition, this study tested the hypothesis that NE activity in the BLA is most important immediately following restraint, by comparing the effects of β-

AR antagonist administered immediately following restraint to the effects of delaying antagonist administration until several hours after restraint. I used the nonspecific β-AR antagonist propranolol at a dose of 0.3 ug/ 0.2 ul. This drug and dose has been demonstrated to create deficits in memory consolidation or reconsolidation when infused into the BLA after training (Debiec & Ledoux, 2004; Roozendaal, Okuda, Van der Zee, & McGaugh, 2006).

There were six groups in this study. Two groups (control; CTL) did not receive any stress prior to day 5, but received daily intra-BLA injections of vehicle (CTL VEH) or propranolol (CTL PROP) on days 1-4. These groups provided a measure of HPA activation to a first, acute exposure to restraint with or without prior drug administration. Four other groups received daily 30 min restraint (repeated restraint; RR) on days 1-4 which were followed by intra-BLA injections. Two of these groups received daily restraint followed immediately by intra-BLA vehicle (RR VEH) or at a 4 hour delay after restraint (RR DELAY VEH) and provided our determination of HPA habituation in the absence of drug administration. Of the remaining two groups, one received daily 30 min restraint immediately followed by intra-BLA propranolol (RR PROP). This group allowed us to test the effect of intra-BLA β−AR blockade after daily repeated restraint on HPA habituation. The final group received daily 30 min restraint followed 4 hours later by intra-BLA propranolol (RR DELAY PROP). The delay group tested whether there was a window of efficacy in which propranolol needed to be administered to alter habituation. On day 5, all animals were restrained and had repeated blood samples taken, and no drug was administered. Thus, all differences between groups on day 5 depended on prior stress and drug exposure on days 1-4.

As will be discussed in more detail below, animals were removed from final analysis in all experiments for several reasons, including insufficient blood sample and misplaced cannulae. Starting n's for Experiment 2.1 were: CTL VEH=11, CTL PROP=13, RR VEH=5, RR PROP=17, RR DELAY VEH=18, RR DELAY PROP=15. The final n's for each group were as follows. ACTH: 0 minutes: CTL VEH=8, CTL PROP=9, RR VEH=4, RR PROP=9, RR DELAY VEH=8, RR DELAY PROP=9. 15 minutes: CTL VEH=7, CTL PROP=6, RR VEH=3, RR PROP=8, RR DELAY VEH=6, RR DELAY

PROP=7. 30 minutes: CTL VEH=7, CTL PROP=5, RR VEH=4, RR PROP=6, RR DELAY VEH=5, RR DELAY PROP=5. 60 minutes= CTL VEH=7, CTL PROP=5, RR VEH=3, RR PROP=5, RR DELAY VEH=9, RR DELAY PROP=7. The n's for the RR VEH animals were unfortunately extremely low, because this group was not included in the first run of this first experiment as the RR DELAY VEH group was considered equivalent. Experiment 2.1 corticosterone was analyzed using repeated measures analysis and thus n for each group is the same at all timepoints: CTL VEH=11, CTL PROP=12, RR VEH=3, RR PROP=8, RR DELAY VEH=10, RR DELAY PROP=9.

Experiment 2.2: What is the effect of intra-BLA β−AR blockade during repeated restraint on the habituation of struggling responses to restraint?

I hypothesized that β−AR activity in the BLA would regulate responses to repeated stressors in general, rather than solely affecting HPA activity. I recently demonstrated that animals that have undergone repeated restraint habituate behaviorally, by reducing the time spent struggling during the first 5 minutes of restraint on day 5 (Grissom, Kerr, & Bhatnagar, 2008; Appendix A) This experiment was designed to examine whether behavioral habituation to restraint would be prevented by post-stress β−AR blockade. The design of this study parallels Experiment 2.1. However, delay groups were not included as the necessity for immediate β−AR blockade after restraint was established in Experiment 2.1 and was not further examined in this dissertation. Thus, there were four groups in this study: CTL VEH, CTL PROP, RR VEH, and RR PROP, all of which received injections immediately after daily restraint, or in the case of CTL groups, at the same time of day. On day 5, all animals were restrained for 30 minutes and were videotaped for subsequent behavioral analysis (described below). No drug was administered on day 5.

Starting n's for Experiment 2.2 were: CTL VEH=24, CTL PROP=26, RR VEH=17, RR PROP=29. Increased numbers of animals per group was necessary for this behavioral measure (e.g. Appendix A) than is necessary for HPA activity. The final n's for this experiment were: CTL VEH=12, CTL PROP=17, RR VEH=15, RR PROP=15.

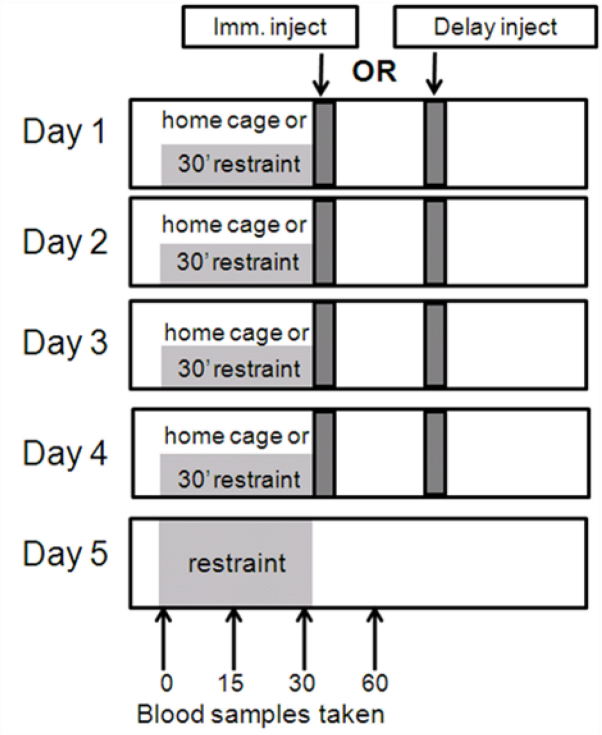

Figure 2.1 Experimental design for Experiment 2.1. After recovery from the implantation of intra-BLA cannulae, animals were either restrained for 30 min or remained in the home cage on days 1-4. Some animals which had been restrained (RR VEH and PROP) received intra-BLA injections immediately following daily restraint. Home cage animals received intra-BLA injections at this time. The remaining repeatedly restrained animals received intra-BLA injections 4 hours following the end of restraint (RR DELAY). On day 5, no drug was administered and all animals were restrained for 30 minutes and had blood samples taken at the times indicated. The design of subsequent experiments in this dissertation is based on this design.

Experiment 2.3: What is the effect of intra-BLA β-AR activation during repeated restraint on HPA habituation to restraint?

This experiment tested the hypothesis that increasing β-AR activation with an agonist after daily restraint could enhance habituation to restraint. Paralleling the deficits in memory consolidation induced by the β-AR antagonist propranolol, the β-AR agonist clenbuterol enhances memory consolidation when infused into the BLA after training (Ferry & McGaugh, 1999; Ferry, Roozendaal, & McGaugh, 1999; McIntyre, Miyashita, Setlow, Marjon, Steward, Guzowski, & McGaugh, 2005; Roozendaal, Quirarte, & McGaugh, 2002; Roozendaal, Schelling, & McGaugh, 2008). Clenbuterol is primarily a β2-AR agonist, although it is believed to have nonspecific or β1-AR agonist effects as

well (Dooley, Bittiger, Hauser, Bischoff, & Waldmeier, 1983; McIntyre, Miyashita, Setlow, Marjon, Steward, Guzowski, & McGaugh, 2005; Roozendaal, Schelling, & McGaugh, 2008). Doses of clenbuterol ranging from 1 ng to 10ng enhance memory consolidation for inhibitory avoidance when infused to the BLA (Ferry & McGaugh, 1999; Ferry, Roozendaal, & McGaugh, 1999; McIntyre, Miyashita, Setlow, Marjon, Steward, Guzowski, & McGaugh, 2005; Roozendaal, Quirarte, & McGaugh, 2002). This produces an inverted-U dose-response curve of NE β-AR agonist effects in the BLA. To explore this dose-response relationship in our paradigm, I elected to use three doses of clenbuterol (1, 3, 10ng) which encompass the range of doses previously shown to be effective at enhancing consolidation.

There were eight groups in Experiment 2.3. Four groups did not receive any stress prior to day 5 but on days 1-4 received daily intra-BLA injections of vehicle (CTL VEH) or clenbuterol (CTL 1ng, CTL 3ng, CTL 10ng). Four additional groups received daily restraint on days 1-4 followed by intra-BLA injection of vehicle (RR VEH) or clenbuterol (RR 1ng, RR 3ng, RR 10ng). All groups were restrained on day 5 and had blood samples taken. I hypothesized that clenbuterol in the BLA after restraint on days 1-4 would enhance habituation on day 5. However, it was possible that habituation would be more rapid in animals receiving clenbuterol. For this reason, I also took blood samples from the RR groups only at the end of restraint on day 3, to compare relative HPA activity between RR animals that received vehicle and those that received clenbuterol. On day three, animals were given intra-BLA injections after blood sampling, and no drug was given on day 5. Thus, all group differences on day 3 and 5 were due to prior stress and drug administration.

Starting n's for Experiment 2.3 were: CTL VEH=16, CTL 1ng=12, CTL 3ng=10, CTL10ng=11, RR VEH=15, RR 1ng=11, RR 3ng=13, RR10ng=13. As above, animals were eliminated from analysis because of insufficient blood sample or misplaced cannulae. Final n's were: Day 3: RR VEH=12, RR 1ng=10, RR 3ng=11, RR10ng=12. Day 5: 0 minutes: CTL VEH=14, CTL 1ng=7, CTL 3ng=9, CTL10ng=8, RR VEH=9, RR 1ng=9, RR 3ng=9, RR10ng=9. 15 minutes: CTL VEH=13, CTL 1ng=8, CTL 3ng=9, CTL10ng=10, RR VEH=9, RR 1ng=7, RR 3ng=7, RR10ng=8. 30 minutes: CTL

VEH=12, CTL 1ng=7, CTL 3ng=8, CTL10ng=8, RR VEH=9, RR 1ng=7, RR 3ng=7, RR10ng=8. 60 minutes: CTL VEH=12, CTL 1ng=7, CTL 3ng=9, CTL10ng=10, RR VEH=8, RR 1ng=7, RR 3ng=7, RR10ng=9.

Surgery

Guide cannulae were implanted into the BLA. Animals were anesthetized with ketamine-xylazine-acepromazine cocktail (77: 1.5: 1.5 mg/kg), then placed in the stereotaxic apparatus. Bilateral cannulae (8mm long, 26 gauge, Plastics One) were directed at the BLA (A/P -2.9 mm; M/L +5.0 mm; D/V -7.6 mm) according to coordinates derived from Paxinos and Watson (2004). Screws were implanted in the skull and cannulae were affixed to these with dental cement. Dummy cannulae were inserted into the guide cannulae to maintain patency. Injector cannulae extended 1mm beyond the tip of the guide cannulae. Animals were monitored during the 4-7 day recovery from surgery prior to beginning experimentation.

Repeated restraint stress

Animals given daily restraint stress (RR groups) were placed in a Plexiglas restrainer for 30 minutes a day in the home room, after which they were returned to their home cage. The restrainers were open-ended Plexiglas cylinders measuring 6.7 cm in diameter and 22.3 cm in length. After the rat was restrained, the restrainer was placed in a clean cage with bedding which held the restrainer in place. Restraint lasted for 30 minutes/day, at which point animals were returned to their home cage. All experiments used a 5-day paradigm, with 4 days of restraint and drug administration, followed by a test restraint on day 5. While this paradigm produces significant habituation (Grissom, Kerr, & Bhatnagar, 2008; Appendix A), it is shorter than repeated restraint paradigms of 8 days typically used in the lab (Bhatnagar, Huber, Nowak, & Trotter, 2002; Jaferi & Bhatnagar, 2006; Jaferi & Bhatnagar, 2007). I elected to shorten the length of the experiment to limit the number of microinjections to the BLA, as repeated microinjections can lead to tissue damage, and to prevent a floor effect of habituation that would obscure β-AR agonist enhancement of habituation.

Drug Administration

The β-AR antagonist propranolol (DL propranolol, Sigma) was dissolved in phosphate buffered saline (PBS) to a final dose of 0.3ug/0.2 ul. The β-AR agonist clenbuterol (Sigma) was dissolved in PBS to a final dose of either 1 ng, 3 ng, or 10 ng per 0.2 ul. Injections of 0.2 ul per side were delivered over a minute and the injector cannulae were left in place an additional 15 seconds to allow for drug diffusion.

Blood Sampling and Radioimmunoassays

Blood samples were obtained from the tail vein, which was nicked at the start of restraint (0 minute blood sample). This method of blood sampling has several advantages over other methods. First, blood samples can be collected rapidly (≤ 1 minute). Elevations in ACTH and corticosterone are not detectable until 2-3 minutes after a manipulation (Vahl, Ulrich-Lai, Ostrander, Dolgas, Elfers, Seeley, D'Alessio, & Herman, 2005), indicating that our 0 minute samples reflect true baselines. Tail nick also allows for the repeated collection of blood samples, unlike cardiac puncture, and does so without the need for additional invasive surgery to implant intravenous catheters. All of these methods have been demonstrated to produce similar values for HPA activity (Vahl, Ulrich-Lai, Ostrander, Dolgas, Elfers, Seeley, D'Alessio, & Herman, 2005) but for the aforementioned reasons tail nick was used to collect blood samples.

Blood samples were obtained repeatedly from animals at the start of restraint (0 minutes), halfway through restraint (15 minutes), at the end of restraint (30 minutes), and 30 minutes after the termination of restraint (60 minutes). Blood was collected on ice into 1.5 ml microcentrifuge tubes containing 10 µl sodium EDTA to prevent coagulation. Whole blood was centrifuged at 2500 rpm for 15 min. The plasma was reserved and frozen at -20°C. Plasma ACTH and corticosterone were measured using kits from MP Biomedicals (Orangeburg, NY). The minimum levels of detection for ACTH and corticosterone were 5.7 pg/ml and 0.6 µg/dl respectively. Intra- and interassay variability was less than 10%.

Behavioral analysis

Struggling behavior in the restrainer (strong mobility), as well as smaller, sub−threshold movements (light mobility) and lack of movement (immobility) was defined, acquired, and analyzed largely as described in Grissom, Kerr, & Bhatnagar (2008). Automatic coding of behavior was analyzed using the EthoVision Pro 3.1 video analysis software (Noldus Information Technology, Leesburg, VA) using the mobility parameter. Briefly, the software is able to give an index of an animal's mobility by detecting the extent of the animal as a field of pixels, and then assessing the percent pixel change between samples of the video. For all automated analyses, the subtraction method of detection was used, detecting all objects different from background.

Detection thresholds were set to ensure that the head and body of the animals were included in observation but the tail was excluded. Three thresholds of pixel change were set in EthoVision to define three different levels of mobility: immobility, mobility (which I label as "light mobility" here to avoid confusion), and strong mobility. Struggling behavior was assessed as strong mobility. "Immobility" was visually indicated by an almost total lack of movement except for breathing was set to register between 0 and 2% pixel change. "Light mobility" was defined by smaller or slower movements of the head occurring throughout the 30 min restraint, including both sniffing and most bouts of grooming, corresponding to between 2 and 6% pixel change. The "strong mobility" parameter was defined by the largest pixel change percentages (greater than 6%, and generally not higher than 15%), which occurred during various struggling/escape behaviors such as chewing on the restrainer, attempts to nose out, back out, or turn around in the restrainer, and rotation within the restrainer. Samples were taken 5 times a second, allowing for fine-tuned distinctions of mobility and conservative estimates of strong mobility. All of these parameters were set based on the criteria determined in Grissom, Kerr, & Bhatnagar, 2008 (Appendix A). Data were analyzed both as 30 min totals and in 5-min bins across the 30 minutes.

Histology

After sample collection on day 5, animals were sacrificed via CO2 and brains were collected and fixed. 30um sections were sliced on a cryostat, dried on slides, and cresyl

violet stained for analysis of cannulae placement. Animals were rejected from analysis if both cannulae were placed 0.5mm or more outside of the BLA. Details of cannulae placement can be found in the Results.

Statistical analyses

For all experiments, all significant effects in an omnibus ANOVA were followed by Fisher's post hoc tests. The significance levels for all tests were set to $p \leq 0.05$.

Experiment 2.1 included six groups, for which ACTH and corticosterone were analyzed at four timepoints during restraint on day 5. The ACTH assay requires a minimum of 25ul of plasma for each sample, but for some animals at some timepoints, there was insufficient plasma available. Because of this, there are missing values for some animals at some timepoints, preventing the use of repeated measures analysis for ACTH in this experiment. Thus ACTH concentration for each timepoint of restraint on day 5 (0, 15, 30, 60 minutes) was analyzed as a separate 2x3 ANOVA for Stress (CTL, RR, RR DELAY) x Drug (VEH, PROP). The corticosterone assay requires a minimum of 2.5ul of plasma for each sample, and thus there were far fewer missing samples for this hormone. As a result, corticosterone concentrations were analyzed in a repeated measures ANOVA for Stress (CTL, RR, RR DELAY) x Drug (VEH, PROP) x Timepoint (0, 15, 30, 60 minutes).

Experiment 2.2 included four groups, for which total immobility, light mobility, and strong mobility (struggling) measured during 30 minute restraint on day 5 were collected. Total time spent in each behavior was analyzed in a 2x2 ANOVA for Stress (CTL, RR) x Drug (VEH, PROP). In addition, immobility, light mobility, and strong mobility measurements were broken into 5 minute bins for a timecourse analysis. Our previous work with this behavior has indicated that significant differences in strong mobility are localized to the first 5-10 minutes (Grissom, Kerr, & Bhatnagar, 2008; Appendix A). Immobility, light mobility, and strong mobility were divided into 5 minute bins across the 30 minute restraint and these data were analyzed in a repeated measures ANOVA for Stress (CTL, RR) x Drug (VEH, PROP) x Timepoint (0-5, 5-10, 10-15, 15-20, 20-25, 25-30 minutes).

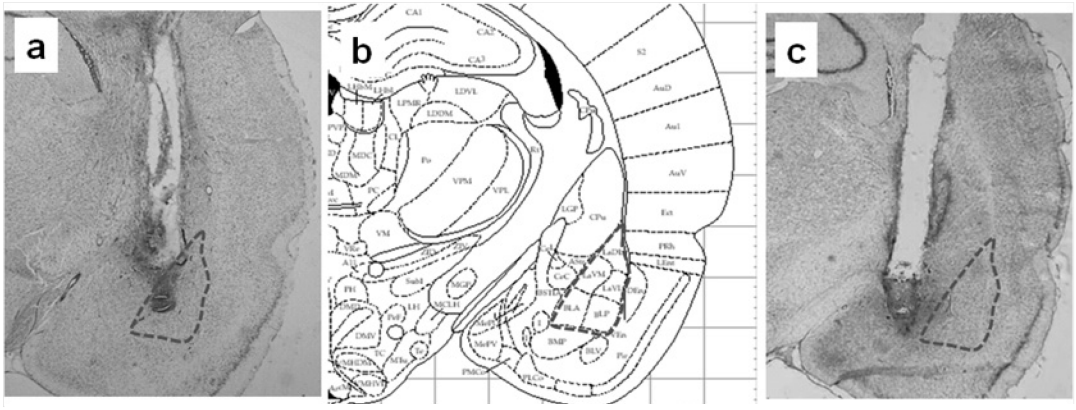

Figure 2.2 Cannulae placements in the BLA. In (a) is an example of a cannula directed at the BLA. In (b) is a plate from Paxinos and Watson (2004) depicting the BLA and surrounding structures at the same approximate level (Bregma -3.3) as in (a) and (c). In (c) is an example of a cannulae which has missed the BLA. The BLA is outlined in (a), (b), and (c) based on the depiction of the nucleus in (b).

Experiment 2.3 contained two days of blood samples, and each day was analyzed separately. As significant habituation was limited to ACTH in Experiment 2.1, only ACTH was analyzed in Experiment 2.3. Blood samples collected on day 3 from the 4 repeatedly restrained (RR) groups were analyzed in a one-way ANOVA based on the dose of clenbuterol (VEH, 1 ng, 3 ng, 10 ng). Blood samples collected on day 5 were analyzed with a 2x4 ANOVA for Stress (CTL, RR) x Drug (VEH, 1 ng, 3 ng, 10 ng). As in Experiment 2.1, missing ACTH values due to sample volume precluded repeated measures analysis of day 5 ACTH. Because it appeared that drug effects of clenbuterol on habituation in RR animals could be obscured by overwhelming main effects of Stress, values from RR groups at the 15 and 30 minute timepoints were also analyzed in one-way ANOVAs, with groups based on the dose of clenbuterol.

2.3 Results

Histology

In all experiments, animals were only kept in analyses if their cannulae were placed in the BLA. The criterion used led to a bilateral hit rate of approximately 50 -75%. Overall, missed cannulae placements were randomly directed, and included those directed medially to the CeA, laterally to the entorhinal cortex, dorsally to the caudate, ventrally to the cortical region below the accessory basolateral amygdalar nucleus, posteriorally to the ventral hippocampus, and anteriorally to the anterior BLA. Figure 2.2 shows an

example of a missed (2.2c) and correctly placed (2.2a) cannulae to the BLA, compared to the appropriate anatomical plate from Paxinos and Watson's atlas of the rat brain (2004; 2.2b).

Experimental groups

Experiment 2.1

The results of Experiment 2.1 are shown in Figure 2.3. No significant effects were observed at 0 minutes into restraint. At 15, 30, and 60 minutes after the onset of restraint on day 5, significant group differences were observed.

At 15 minutes, there was a Main Effect of Stress ($F(2,29)=4.1$, $p\leq0.05$) and a significant Stress x Drug Interaction ($F(2,29)=3.3$, $p\leq0.05$). Posthoc tests indicated that CTL VEH animals, which received no restraint prior to day 5, had ACTH values significantly greater than those seen in repeatedly restrained animals given either immediate (RR VEH, $p\leq0.05$) or delayed (RR DELAY VEH, $p\leq0.01$) vehicle, or those repeatedly restrained animals given delayed administration of propranolol (RR DELAY PROP, $p\leq0.01$). This indicates significant habituation in the RR VEH, RR DELAY VEH, and RR DELAY PROP groups at 15 minutes into restraint as compared to CTL VEH. Similarly, RR PROP animals, which received propranolol in the BLA immediately after daily restraint, had significantly greater ACTH values at 15 minutes into restraint than RR VEH ($p\leq0.05$), RR DELAY VEH ($p\leq0.01$), and RR DELAY PROP ($p\leq0.05$) animals. RR PROP animals were not different from CTL VEH or CTL PROP. This indicates that administration of propranolol into the BLA prevented habituation to repeated restraint at the 15 minute timepoint.

At 30 minutes into restraint on day 5, a similar pattern was observed. There was a Main Effect of Stress ($F(2,26)=7.0$, $p\leq0.01$) and a Stress x Drug Interaction ($F(2,26)=3.5$, $p\leq0.05$). Posthoc tests indicated that CTL VEH animals had ACTH values significantly greater than RR DELAY VEH ($p\leq0.01$) and RR DELAY PROP ($p\leq0.01$). The difference between CTL VEH and RR VEH animals had a p value of 0.06. CTL PROP animals had ACTH concentrations significantly higher than RR DELAY VEH ($p\leq0.05$) and tended to be higher than RR DELAY PROP ($p=0.07$). This indicates that a significant habituation

of ACTH activity was observed in both delay groups, and this habituation was nearly significant in animals that received immediate post-restraint injections of vehicle. RR PROP animals had significantly elevated ACTH values at 30 minutes into restraint compared to RR VEH ($p \leq 0.005$), RR DELAY VEH ($p \leq 0.001$), and RR DELAY PROP ($p \leq 0.001$). RR PROP animals were not significantly different from CTL VEH and CTL PROP. These results indicate that habituation to repeated restraint at the 30 minute timepoint was prevented by administration of propranolol into the BLA immediately after daily restraint.

At the 60 minute timepoint on day 5, after 30 minutes of recovery from repeated restraint, a main effect of treatment was observed ($F(2, 30)=5.5$, $p \leq 0.01$). Posthoc tests indicate that overall, repeatedly restrained groups given immediate post-restraint injections had higher recovery values than those animals that received delayed post-restraint injections ($p \leq 0.05$). No other significant effects were observed at any timepoint.

Corticosterone concentrations are shown in Figure 2.3 c and d. Repeated measures ANOVA for Stress (CTL, RR, RR DELAY) x Drug (VEH, PROP) x Timepoint (0, 15, 30, 60) revealed a Main Effect of Timepoint ($F(3,141)=98.5$, $p \leq 0.0001$), and a trend towards a Main Effect of Treatment ($F(2,47)=2.8$, $p=0.07$). Posthoc tests indicated that 15, 30, and 60 minute values were significantly elevated compared to 0 minute values ($p \leq 0.0001$), and 30 minute values were significantly elevated compared to 15 and 60 minute values ($p \leq 0.0001$). These results indicate an overall activation of corticosterone in response to restraint. The direction of the trend in the Treatment effect was a significant decrease in RR DELAY groups compared to CTL groups, indicating a trend towards habituation in RR DELAY groups. No other significant effects of corticosterone were observed.

In sum, the results of Experiment 2.1 indicate that administration of the β-AR antagonist propranolol into the BLA immediately after daily restraint (RR PROP) prevented the habituation of ACTH activity during exposure to a 5^{th} restraint. This effect did not occur if propranolol was not administered within 4 hours of the daily restraint, as RR DELAY PROP animals exhibited significant habituation at 15 and 30 minutes.

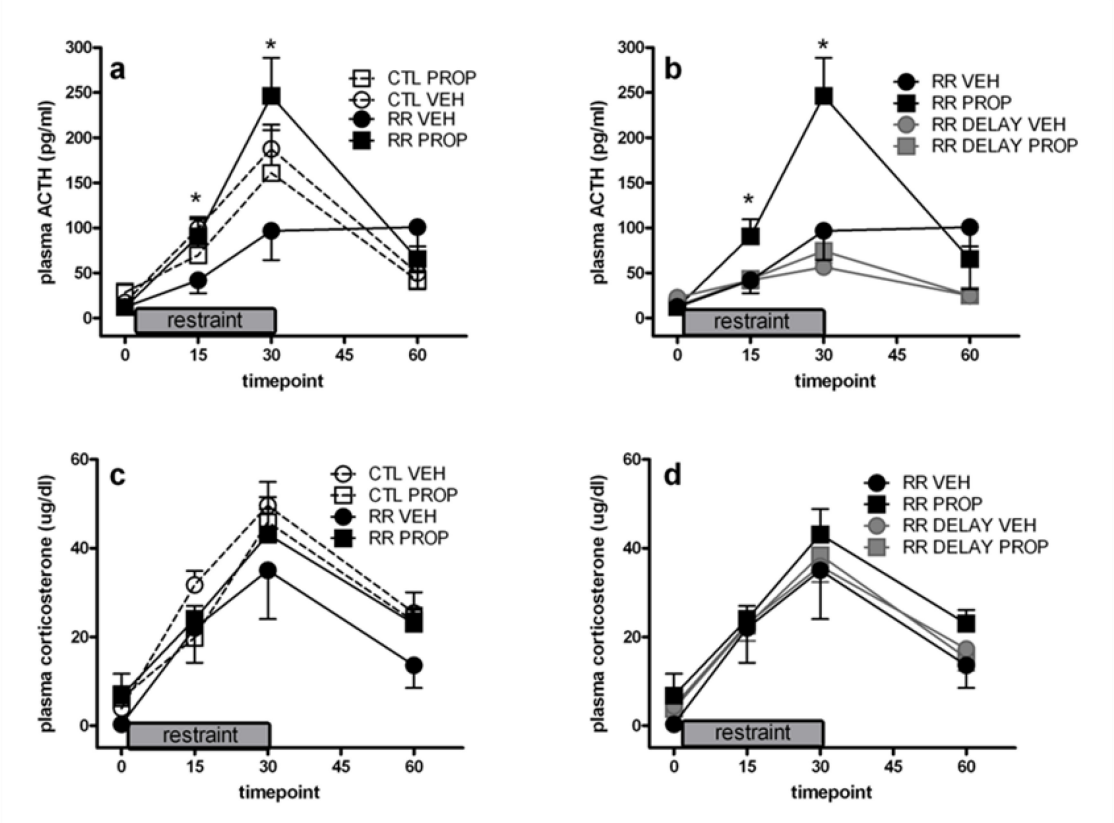

Figure 2.3 HPA activity to restraint after repeated restraint and intra-BLA propranolol in Experiment 2.1. In (a) and (b) are ACTH values for restraint on day 5, and in (c) and (d) are corticosterone values. The six groups are divided between two graphs for clarity, and RR VEH and RR PROP are included in both for comparsion. (a) Compared to CTL VEH, RR VEH is significantly (*) habituated at 15 minutes, and trends ($p \leq 0.06$) towards significant habituation at 30 minutes. Compared to RR PROP, RR VEH is significantly habituated at 15 and 30 minutes. (b) Compared to RR PROP, all other groups in this graph are significantly habituated at 15and 30 minutes into restraint. These results indicate that immediate post-restraint propranolol into the BLA on days 1-4 prevented the habituation of ACTH activity on day 5 in RR PROP animals, but that delayed post-restraint administration of propranolol did not prevent habituation in RR DELAY PROP animals. There were no significant effects of corticosterone in (c) and (d). Please see text for full list of significant comparisons. Figures are means ± SEM.

Experiment 2.2

The results of Experiment 2.2 are depicted in Figure 2.4. 2x2 ANOVA for Stress (CTL, RR) x Drug (VEH, PROP) ANOVA were first conducted on total times spent immobile, lightly mobile, and strongly mobile. 2x2 ANOVA for total time spent immobile revealed a significant Main Effect of Stress ($F(1,55)=6.0$, $p \leq 0.01$) and a significant Interaction ($F(1,55)=3.7$, $p \leq 0.05$). There was a similar pattern for total time spent lightly mobile (Main Effect of Stress: $F(1,55)=5.6$, $p \leq 0.05$; Interaction: $F(1,55)=3.9$, $p \leq 0.05$). In both total time spent immobile and lightly mobile, Fishers posthoc tests indicated that RR VEH animals had significantly higher immobility ($p \leq 0.05$), and significantly lower light

mobility ($p \leq 0.005$), than CTL VEH animals. 2x2 ANOVA for total time spent strongly mobile revealed a trend towards a Main Effect of Stress ($F(1,55)=2.7$, $p=0.1$) indicating that RR groups tended to be less strongly mobile, or spend less time struggling, than CTL groups.

As previous results in our laboratory confirmed that habituation of struggling behavior occurred within the first 5-10 minutes of restraint, I next conducted repeated measures ANOVA on Stress x Drug x Timepoint(0-5, 5-10, 10-15, 15-20, 20-25, 25-30) for immobility, light mobility, and strong mobility. The key measure of struggling is strong mobility.

Repeated measures ANOVA for strong mobility revealed a Main Effect of Timepoint ($F(5,275)=38.6$, $p \leq 0.0001$) and Interaction Effects of Timepoint x Stress ($F(5,275)=5.7$, $p \leq 0.0001$) , Timepoint x Drug ($F(5,275)=3.2$, $p \leq 0.01$) , and Timepoint x Stress x Drug ($F(5,275)=2.5$, $p \leq 0.05$). Posthoc tests for the Timepoint by Stress by Drug interaction revealed many significant comparisons. Confirming the hypothesis of this experiment, the CTL VEH ($p \leq 0.0001$), CTL PROP ($p \leq 0.0001$), and RR PROP ($p \leq 0.001$) groups spent significantly more time strongly mobile during the first 5 minutes of restraint than RR VEH animals, indicating 1) a significant habituation of struggling behavior in RR VEH animals as compared to CTL groups and 2) a blockade of habituation in RR PROP animals as compared to RR VEH animals. CTL VEH ($p \leq 0.001$), CTL PROP ($p \leq 0.005$), and RR PROP ($p \leq 0.05$) groups all spent significantly more time strongly mobile during the first 5 minutes of restraint than during the rest of their restraint period, replicating our finding of within-restraint reductions in struggling behavior (Grissom, Kerr, & Bhatnagar, 2008; Appendix A).

Repeated measures ANOVA for immobility revealed a Main Effect of Stress ($F(1,55)=5.9$, $p \leq 0.01$), an Interaction between Stress and Drug ($F(1,55)=4.0$, $p \leq 0.05$), a Main Effect of Timepoint ($F(5, 275)=33.2$, $p \leq 0.001$), and a Drug x Timepoint Interaction ($F(5,275)=2.6$, $p \leq 0.05$). Posthoc tests on the Stress x Drug interaction indicated that RR VEH was significantly more immobile than CTL VEH ($p \leq 0.05$). Posthoc tests on the Drug x Timepoint interaction indicated that in general, VEH and PROP animals were equivalent at each timepoint, increasing in immobility over the 30 minute restraint, but

the rate of increase was slower in VEH animals than PROP animals. Thus, VEH animals at 0-5 minutes were different from all other VEH timepoints ($p \leq 0.05$). VEH animals at 5-10 minutes were only less immobile than the 25-30 minute timepoint ($p \leq 0.05$), and the remaining VEH timepoints were not different from each other. In contrast, the rate of change was greater per timepoint for PROP animals. PROP at 0-5 minutes were significantly less immobile than PROP at all other timepoints ($p \leq 0.0005$), PROP at 5-10 minutes were significantly less immobile than at 20-25 and 25-30 minutes ($p \leq 0.005$), and PROP at 10-15 minutes and 15-20 minutes were significantly less immobile than PROP at 25-30 minutes ($p \leq 0.05$). Thus, VEH animals increased their immobility over time less than PROP animals.

Repeated measures ANOVA done on light mobility revealed a similar pattern of results, but inverted as light mobility decreases over the restraint period, while immobility increases. There was a Main Effect of Stress ($F(1,55)=5.5$, $p \leq 0.05$) and a significant Interaction between Stress and Drug ($F(1,55)=4.1$, $p \leq 0.05$). There was also a Main Effect of Timepoint ($F(5,275)=23.7$, $p \leq 0.001$). The Stress x Drug interaction indicated that RR VEH animals were less lightly mobile than CTL VEH ($p \leq 0.005$). The Main Effect of Timepoint indicated that over time, light mobility increased over the 30 minute restraint period.

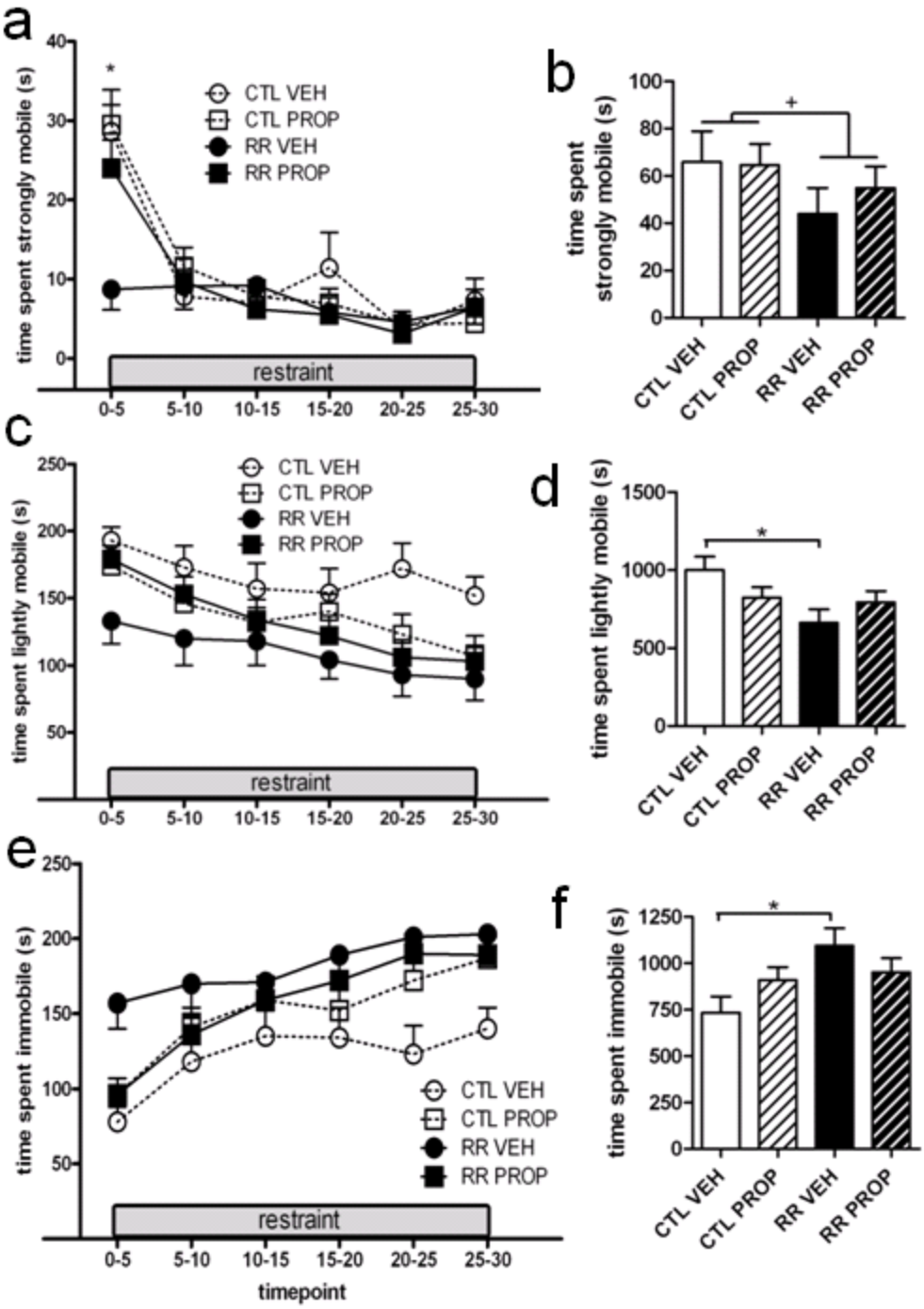

Figure 2.4 Struggling during restraint after repeated restraint and intra-BLA propranolol in Experiment 2.2. Figures (a) and (b) depict strong mobility, (c) and (d) depict light mobility, and (e) and (f) depict immobility. Figures (a), (c), and (e) depict these measures in 5 minute bins across the 30 minute restraint on day 5, and (b), (d), and (f) depict the total time spent in these behaviors. In (a), compared to all other groups, RR VEH animals show a significant (*) habituation of strong mobility (the struggling response). This indicates that intra-BLA propranolol prevented the habituation of struggling behavior in RR PROP animals. See text for full list of significant comparisons. Figures depict means ± SEM.

In sum, the results of Experiment 2 indicate that administration of β-AR antagonist into the BLA after daily restraint appears to attenuate the habituation of struggling behavior to a 5th restraint.

Experiment 2.3

The results for Experiment 2.3 are shown in Figure 2.5. Because significant effects were limited to ACTH in Experiment 2.1, only ACTH was measured in Experiment 2.3. On day 3, repeatedly restrained (RR) animals had blood samples taken at the end of 30 minute restraint. One way ANOVA revealed a significant difference between groups ($F(3,41)=3.2$, $p\leq0.05$). Posthoc tests indicated that compared to RR VEH animals, RR 1ng animals had significantly reduced ACTH activity at the end of restraint on day 3 ($p\leq0.005$). RR 3ng animals exhibited a trend towards reduced ACTH activity on day 3 as compared to RR VEH animals ($p=0.06$). RR 1ng animals also exhibited significantly reduced ACTH activity on day 3 as compared to RR 10ng animals ($p\leq0.05$). Thus, 1ng clenbuterol after 2 days of restraint significantly reduced ACTH activity to restraint on day 3.

On day 5, all animals were restrained for 30 minutes. No effects were observed at 0 minutes. At 15 and 30 minutes into restraint on day 5, a Main Effect of Stress was observed on ACTH concentration (15 minutes: $F(1,63)=8.2$, $p\leq0.01$; 30 minutes: $F(1,59)=18.5$, $p\leq0.0001$). All RR groups had significantly reduced ACTH activity compared to all CTL groups. Clenbuterol was hypothesized to enhance habituation, but it appeared as though these enhancing effects could be masked by the much larger effects

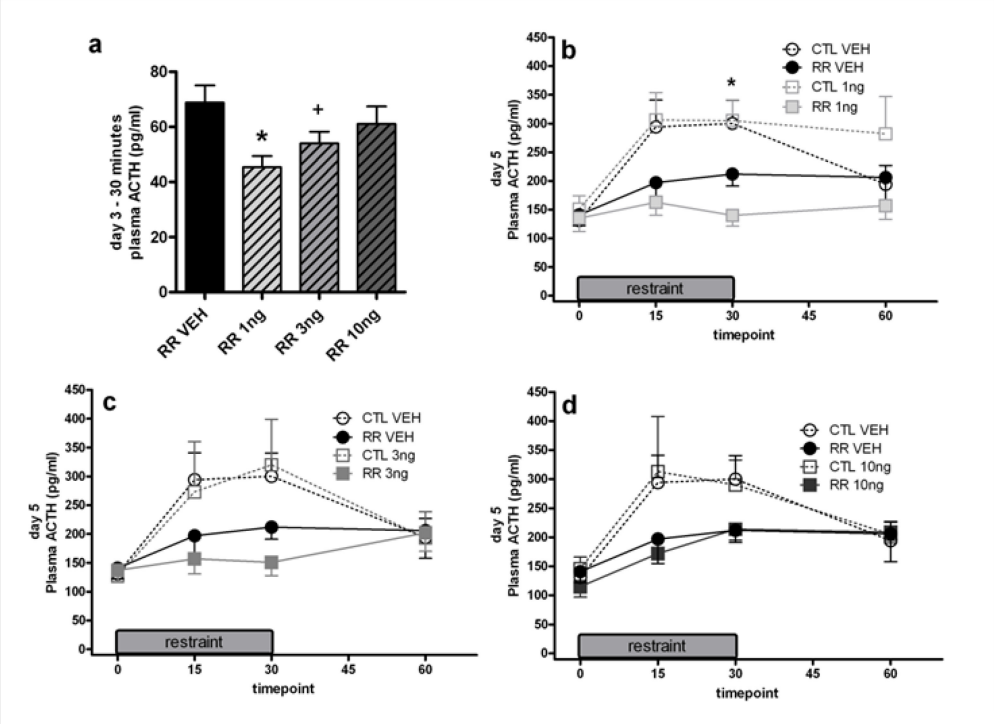

Figure 2.5 ACTH activity to restraint after repeated restraint and intra-BLA clenbuterol in Experiment 2.3. In (a) are ACTH concentration for RR groups only at the end of restraint on day 3. In (b), (c), and (d) are ACTH concentration in response to restraint on day 5. CTL VEH and RR VEH groups are included on all three graphs for comparison. In (a), 1ng clenbuterol after restraint on days 1 and 2 resulted in a significant (*) reduction in ACTH concentrations at the end of day 3 compared to vehicle. The 3ng dose tended to be lower than vehicle on day 3 (+, $p \leq0.06$). At the end of 30 min restraint on day 5 in (b), comparison of the repeatedly restrained groups indicated that the 1ng dose of clenbuterol (RR 1ng) resulted in reduced ACTH concentration compared to repeatedly restrained animals which received vehicle after daily restraint (RR VEH). In sum, 1ng clenbuterol in the BLA after daily restraint enhanced habituation to restraint. See text for a full list of significant comparsions. Figures depict means ± SEM.

of Stress. Thus, I conducted one-way ANOVAs for ACTH concentrations in RR groups only on 15 and 30 minutes. No significant effects were seen on ACTH concentrations at 15 minutes in RR groups only. At 30 minutes into restraint on day 5, a significant difference between RR groups was observed ($F(3,26)=3.0$, $p\leq0.05$). Posthoc tests

indicated that as on day 3, RR 1ng animals exhibited significantly reduced ACTH compared to RR VEH ($p \leq 0.05$) and/or RR 10ng ($p \leq 0.05$) animals on day 5.

In sum, the results of Experiment 3 indicate that administration of 1ng of the β-AR agonist clenbuterol into the BLA after daily restraint leads to a reduction in ACTH activity elicited by restraint day 3 as compared to repeatedly restrained animals that received vehicle in the BLA, and a small but significant enhancement of habituation on day 5. Higher doses were not effective at enhancing habituation, indicating an inverted-U shaped dose response curve. Thus, 1ng clenbuterol administered in the BLA after daily restraint enhances habituation.

2.4 Discussion

The current experiments were designed to test a novel role for the BLA in regulating the acquisition of habituation to repeated restraint. The BLA is altered in structure and function as a result of repeated stressor exposure (Bhatnagar & Dallman, 1998; Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002) and it is well known to regulate the acquisition of learned responses to aversive stimuli (Maren, 2005; McGaugh, 2004; Sandi & Pinelo-Nava, 2007). In many cases, the role of the BLA in the acquisition of emotional memory is mediated by NE β-AR signaling in this region (Debiec & Ledoux, 2004; Roozendaal, Okuda, Van der Zee, & McGaugh, 2006; Roozendaal, Okuda, de Quervain, & McGaugh, 2006). Thus, in the current experiments I tested a role for this signaling in mediating the acquisition of stress experience-dependent changes in HPA and behavior.

In the first experiment, I hypothesized that in concordance with reports on other paradigms (Miranda, Rodri Guez-Garci, Reyes-Lopez, Ferry, & Ferreira, 2008; Roozendaal, Hahn, Nathan, de Quervain, & McGaugh, 2004; Roozendaal, Okuda, Van der Zee, & McGaugh, 2006), intra-BLA β-AR antagonist propranolol infused immediately after restraint on days 1-4 would prevent the habituation of HPA activity to a test restraint on day 5. In response to restraint on day 5, I saw significant habituation of ACTH activity in repeatedly restrained animals given intra-BLA vehicle at 15 and 30 minutes into a test restraint compared to control animals. As hypothesized, the effect of intra-BLA β-AR blockade via propranolol immediately after daily restraint was to

prevent this habituation from being observed at 15 and 30 minutes into the restraint on day 5. In contrast to the effects of immediate post-restraint intra-BLA β-AR blockade, administration of β-AR antagonist 4 hours after daily restraint did not affect habituation to repeated restraint. This result indicates that there is a critical window during the post-restraint period where NE signaling exerts its effects in the BLA to alter habituation. During this period, activation of β-AR appears to be required for habituation to occur. However, at the 60 minute stress recovery timepoint, I found that animals that had received immediate post stress injections, regardless of whether these injections were vehicle or β-AR antagonist, had elevated ACTH activity relative to those animals that received delayed post-restraint injections. This effect seems driven by elevated ACTH concentration in repeatedly restrained animals that received immediate post-restraint vehicle. It is possible that this result reflects a spike in HPA activity after restraint on day 5 due to a violation of expectancy in the immediate groups, which would normally have received an injection during the 30 minute recovery period. However, such a spike is not observed at the 60 minute timepoint in Experiment 3, in which RR animals also received injections immediately post-restraint. As the error in the RR VEH group at 60 minutes in Experiment 1 is very high, and this effect is not replicated in Experiment 3, I believe this spike is not a meaningful finding. Thus, the overall interpretation of the experiment remains that activity of β-AR in the BLA are required in the immediate post-restraint period to allow habituation to repeated restraint to occur.

A potential caveat to this interpretation, however, is the lack of significant effects on corticosterone at any timepoint. Specifically, habituation was not observed in corticosterone in repeatedly restrained animals given intra-BLA vehicle. There may be an overall ceiling effect in corticosterone in RR VEH groups, which obscured any differences that might exist between these animals and those that received restraint followed by propranolol. Such a ceiling effect in corticosterone but not ACTH is not unheard of in the stress literature and there are several candidate explanations for this phenomenon. First, it is possible that 5 days of 30 minute restraint is insufficient to reliably induce significant habituation of corticosterone activity. A 5 day paradigm was selected on the basis of pilot testing as the least number of days needed to reliably elicit

habituation of ACTH activity to limit the number of daily microinjections delivered to the BLA. According to the current literature it appears that it takes a minimum of 8 exposures to restraint for corticosterone concentrations to significantly habituate (Ma & Lightman, 1998). Mechanistically, cyclic AMP activation in the adrenal, the rate-limiting step for corticosterone synthesis and release, plateaus at a relatively low concentration of ACTH (Dallman, Akana, Cascio, Darlington, Jacobson, & Levin, 1987) such that large differences in ACTH secretion can lead to little or no difference in corticosterone concentrations. Secondly, the release of corticosterone is under control of not only ACTH but also direct innervation by the splanchnic nerve, which as part of the autonomic nervous system is also activated under conditions of stress (Ehrhart-Bornstein & Bornstein, 2008; Ulrich-Lai, Arnhold, & Engeland, 2006). Finally, repeated stressors can lead to changes in adrenal sensitivity to ACTH (Armario, Restrepo, Castellanos, & Balasch, 1985; Armario, Hidalgo, & Giralt, 1988). These factors suggest that corticosterone is less sensitive a measure of habituation processes than ACTH, which is elicited exclusively in response to secretagogue release from the hypothalamus (Armario, 2006). I believe that were the experiment to be extended to eight or more days of restraint, intra-BLA propranolol would result in a prevention of habituation in corticosterone as well as ACTH.

In the second experiment, I hypothesized that intra-BLA β-AR blockade after restraint on days 1-4 would prevent the habituation of struggling behavior, measured as strong mobility, to a test restraint on day 5. In concordance with our previous demonstrations of the habituation of struggling (Grissom, Kerr, & Bhatnagar, 2008; Appendix A), repeatedly restrained animals given intra-BLA vehicle in Experiment 2.2 demonstrated a significant reduction in strong mobility during the first 5 minutes of restraint on day 5 compared to control animals. However, intra-BLA β-AR antagonist after daily restraint prevented this habituation in behavior during the first 5 minutes of restraint on day 5. In our previous demonstrations of behavioral habituation to restraint, the first 5 minutes was the crucial time during which all significant differences in strong mobility between repeatedly restrained and control animals were observed. The difference at this timepoint in previous experiments was large enough to result in a significant difference in strong mobility across the entire 30 minute restraint as well. While I did not see a difference in

total strong mobility in Experiment 2.2, the mean times spent struggling for habituated and nonhabituated animals in this experiment are very similar to our previous observations (Appendix A), indicating that perhaps the stereotaxic surgery and/or injections increased variability in the current experiment. Overall, the results of the first two experiments demonstrate that intra-BLA administration of a β-AR antagonist after exposure to a stressor prevents experience-dependent changes in HPA and behavioral activity when exposed to that stressor in the future.

Based on the findings that β-AR antagonist in the BLA after daily restraint prevents habituation, I hypothesized that a β-AR agonist infused into the BLA after daily restraint should enhance habituation of HPA activity. Due to the inverted-U dose-response relationship seen with β-AR agonists in other paradigms, I tested a range of doses of the β-AR agonist clenbuterol which have all been effective at enhancing memory consolidation when infused in the BLA (Ferry & McGaugh, 1999; Ferry, Roozendaal, & McGaugh, 1999; McIntyre, Miyashita, Setlow, Marjon, Steward, Guzowski, & McGaugh, 2005; Roozendaal, Quirarte, & McGaugh, 2002). At the end of restraint on day 3, a 1ng dose of clenbuterol lead to significantly lower ACTH activity than that seen in repeatedly restrained animals given vehicle or 10ng clenbuterol. At the end of restraint on day 5, repeatedly restrained groups had significantly habituated HPA activity. A separate analysis of the repeatedly restrained groups on day 5 indicated that the 1ng dose of clenbuterol lead to significantly reduced ACTH activity at the 30 minute timepoint compared to animals that received vehicle or 10ng clenbuterol. While this effect was limited to the 30 minute timepoint, this timepoint appears to reflect the peak of ACTH concentrations in response to 30 minute restraint in the current experiments. Thus, differences at 30 minutes may be most reflective of group differences in restraint-induced HPA activity. Overall, Experiment 2.3 indicates that post-restraint infusion of 1ng of the β-AR agonist clenbuterol in the BLA induces a modest but significant enhancement of habituation.

While at first it may appear counterintuitive that the lower doses of clenbuterol were more effective at enhancing habituation than the higher dose, this result fits well with the literature, which has repeatedly demonstrated an inverted-U dose-response function for

β-AR activity in the BLA and memory consolidation. This function predicts that an optimum level of β-AR activation would lead to most rapid and complete habituation, but that activation that is lower, as would be induced by the β-AR antagonist propranolol, or higher, as would be induced by high doses of the β-AR agonist clenbuterol, would both lead to attenuations in habituation. This leads to the interesting prediction that a very high dose of clenbuterol in this paradigm could be as effective at preventing habituation as propranolol was in Experiment 1. Such a pattern might also explain the discrepancies in the literature regarding an effective dose of β-AR agonist to infuse in the BLA to alter memory consolidation. Differences in individual experimental protocol may interact to produce different levels of baseline noradrenergic activity in the BLA between experiments, which would alter the dose of clenbuterol necessary to reach an optimum level of β-AR activation.

Overall, the current results indicate 1) that blocking β-AR in the BLA after daily restraint prevents the habituation of HPA activity and struggling behavior to restraint; 2) β-AR blockade must occur within 4 hours of daily restraint, indicating that the actions of NE in the BLA which alter habituation occur within these 4 hours; 3) that administration of a β-AR agonist after daily restraint enhances habituation to restraint. These results make a strong case that noradrenergic activity in the BLA immediately following a stressor is important for the development of habituation of HPA and behavioral responses to repeated stressors. However, this interpretation of the results is predicated on the accuracy of the intra-BLA drug administration in the current experiments. In particular, given the important role of the central amygdala (CeA) in regulating HPA activity and behavior (Cecchi, Khoshbouei, & Morilak, 2002; Keen-Rhinehart, Michopoulos, Toufexis, Martin, Nair, Ressler, Davis, Owens, Nemeroff, & Wilson, 2008; Lang & Davis, 2006), and as an afferent of the BLA (Lang & Davis, 2006), one might argue that the effects of the β-AR antagonist or agonist in the current experiments could be occurring in the CeA instead of, or in addition to, the BLA. This is unlikely to be the case. Autoradiography for β1- and β2-AR in the rat brain detected little or no β-AR in the CeA (Rainbow, Parsons, & Wolfe, 1984). By way of addressing this potential caveat, in Figure 2.6 I show the data from animals in Experiment 2.1 in the RR PROP group

which were determined not to have cannulae directed at the BLA. These missed placements included many medial misses, which directed the cannulae at the CeA. Figure 2.6 shows that the "missed placements" group showed a clear habituation of ACTH activity to repeated restraint, despite having received propranolol injections immediately after restraint into regions other than the BLA. Thus, I believe that the effects of the β-AR manipulations in the current studies are occurring specifically in the BLA.

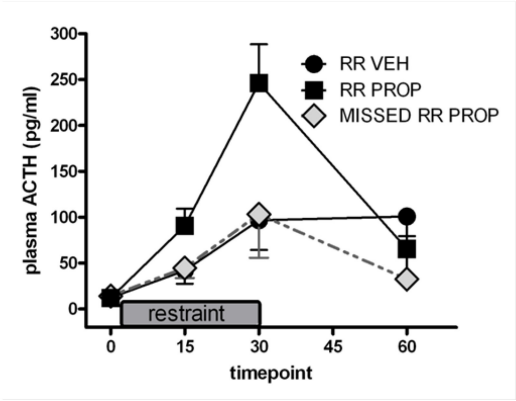

Figure 2.6 Results of missed BLA placements in Experiment 2.1. Those animals in the RR PROP group which did not have cannulae directed at the BLA were excluded from analysis. These excluded animals, collected as the MISSED RR PROP group, demonstrate habituation of ACTH concentrations in response to restraint on day 5. Figure depicts means ± SEM.

The results of the current studies indicate that β-AR activation in the BLA immediately after daily restraint is necessary for the habituation of HPA activity and struggling behavior to occur to restraint. This finding links several literatures together, including the effects of repeated stressors on BLA structure and function (Bhatnagar & Dallman, 1998; Bhatnagar, Vining, & Denski, 2004; Mitra, Jadhav, McEwen, Vyas, & Chattarji, 2005; Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002; Vyas, Pillai, & Chattarji, 2004; Vyas, Jadhav, & Chattarji, 2006), the role of the BLA in memory consolidation (McGaugh, 2004), and the role of NE in regulating HPA activity (Cecchi, Khoshbouei, & Morilak, 2002; Ma & Morilak, 2005; Pardon, Gould, Garcia, Phillips, Cook, Miller, Mason, & Morilak, 2002; Pardon, Ma, & Morilak, 2003). NE signaling in the BLA is important to adaptation to repeated stressors, linking stress response adaptation to the learning and memory literature, and linking the BLA to the existing literature of the role of NE in stress regulation. These findings also raise a number of questions, all centered on discovering what changes take place in the brain because of chronic stressors that are

necessary for habituation. First, there are a number of stress-regulatory brain regions that can undergo changes in gene expression in response to repeated stressors, some of which may be particularly relevant to habituation. It would be interesting to know whether these changes in gene expression are prevented or diminished by following daily stress by β-AR blockade, as that would highlight changes that are necessary for habituation to occur. On a different tack, it would be beneficial to understand the biochemical cascades occurring in the BLA because of repeated stressors, and to learn which of these were being blocked by propranolol, to begin to shed light on how stressors, in combination with β-AR signaling in this region, can exert profound effects on physiology and behavior. These two topics with be the subjects of the subsequent chapters.

Chapter 3.

Gene expression in stress-regulatory brain regions is altered by repeated restraint and intra-BLA β−AR blockade

3.1 Introduction

In Chapter 2, I found that β−AR activation in the BLA after daily repeated restraint influenced the habituation in ACTH and struggling responses seen to restraint. In particular, blockade of β−AR activation in the BLA immediately after daily restraint prevented the habituation of HPA activity and struggling behavior. These results raise the question of what effects β−AR signaling in the BLA has on the activity of stress-response-regulatory brain regions. If stressor-induced changes in the activity of these regions are prevented by β-AR blockade in the BLA, it would strongly suggest that these changes reflect crucial mechanisms of habituation.

Repeated stressor exposure alters patterns of gene expression in many brain regions, described in further detail below. These changes include increased mRNA expression of one of the key stress-regulatory neuropeptides in the hypothalamus, which is directly involved in stimulating the HPA axis. There is also evidence of decreased mRNA expression of brain-derived neurotrophic factor (BDNF) in the BLA and hippocampus in repeatedly stressed animals, which may be involved in changes in neural plasticity related to the aversive event.

Changes in the expression of hypothalamic neuropeptides by repeated stressors represent a relatively straightforward mechanism of HPA regulation by prior stressor exposure. Medial parvocellular neurons in the paraventricular nucleus of the hypothalamus (mpPVN) secrete the neuropeptides CRF and AVP peptide as the first step of HPA activation (Lightman, 2008; Sapolsky, Romero, & Munck, 2000). The expression of mRNA for AVP is tonically regulated by prior repeated stressors. Repeated exposure to the mild-to-moderate stressor of repeated restraint such as that used in Chapter 2 leads to

a reliable increase in tonic AVP mRNA expression in the mpPVN (Aubry, Bartanusz, Jezova, Belin, & Kiss, 1999; Ma & Lightman, 1998; Ma, Levy, & Lightman, 1997; Pinnock & Herbert, 2001). No change is seen in CRF mRNA expression in the mpPVN under these conditions (Ma & Lightman, 1998; Ma, Levy, & Lightman, 1997). The repeated stressor-induced increase in AVP in the mpPVN is at least in part due to increase co-expression of AVP in CRF-expressing neurons (Aubry, Bartanusz, Jezova, Belin, & Kiss, 1999; De Goeij, Jezova, & Tilders, 1992). The increase in AVP expression is thought to potentiate the effects of CRF and maintain HPA axis responsiveness to a novel stressor in the face of stimuli that lead to decreased HPA activity, such as habituation, glucocorticoid negative feedback, and pituitary desensitization (Aguilera, Subburaju, Young, & Chen, 2008; Ma & Lightman, 1998). Thus, increased AVP mRNA in the mpPVN represents an important change in neural regulation of HPA activity that is induced during habituation to repeated stressors, and reflects changes in activity of PVN inputs.

Recent research has focused on the learning- and stressor-induced changes in mRNA of the neurotrophin BDNF in limbic regions, particularly focusing on the hippocampus but also including the BLA. Increased BDNF expression in the hippocampus is required for the consolidation of contextual fear conditioning (Lee, Everitt, & Thomas, 2004). In the BLA, BDNF mRNA was increased after exposure to shocks paired with odor, but not after unpaired shocks (Jones, Stanek-Rattiner, Davis, & Ressler, 2007). BDNF has also been shown to be important for the effects of stressor exposure on depressive-like symptoms. Increased BDNF in the hippocampus leads to antidepressant effects (Govindarajan, Rao, Nair, Trinh, Mawjee, Tonegawa, & Chattarji, 2006) and while repeated stressors induce a downregulation of BDNF mRNA in the hippocampus and BLA (Bergström, Jayatissa, Mørk, & Wiborg, 2008; Pizarro, Lumley, Medina, Robison, Chang, Alagappan, Bah, Dawood, Shah, Mark, Kendall, Smith, Saviolakis, & Meyerhoff, 2004; Tsankova, Berton, Renthal, Kumar, Neve, & Nestler, 2006), antidepressant treatment can prevent this downregulation (Balu, Hoshaw, Malberg, Rosenzweig-Lipson, Schechter, & Lucki, 2008; Govindarajan, Rao, Nair, Trinh, Mawjee, Tonegawa, & Chattarji, 2006; Tsankova, Berton, Renthal, Kumar, Neve, & Nestler, 2006). Thus, BDNF expression in the hippocampus and BLA may integrate the effects of stressors on

mood and memory. The reduction of BDNF mRNA expression in these regions caused by repeated stressors may be one mechanism involved in the habituation of which HPA and behavioral responses.

The blockade of ACTH and behavioral habituation to repeated restraint by post-restraint infusions of β−AR antagonist into the BLA raises the question of how the function of stress-response-regulatory brain regions are altered by repeated stress and β−AR blockade. First, repeated stressors increase AVP mRNA in the PVN. Is this increase seen in repeatedly restrained animals in the current paradigm, and does intra-BLA β−AR blockade via propranolol prevent this increase? Second, repeated stressors decrease BDNF mRNA in the BLA and hippocampus. Is this decrease seen in repeatedly restrained animals in the current paradigm, and is this decrease prevented by intra-BLA propranolol? Evidence suggests that intra-BLA manipulations, including β-AR activation, affect hippocampal neuronal activity and may be a mechanism by which intra-BLA β-AR effects exert changes on behavior (Akirav & Richter-Levin, 2006; Tsoory, Vouimba, Akirav, Kavushansky, Avital, & Richter-Levin, 2008). I hypothesized that in the current experiment, repeated restraint would replicate previous findings of mRNA regulation in the PVN, BLA, and hippocampus, but that β−AR blockade would prevent these changes.

3.2 Methods

Animals and Surgery

Experimental animals were obtained and housed, and intra-BLA cannulae were implanted, as described in Chapter 2.

Experimental design

There were four groups in this study, in a 2x2 design: CTL VEH, CTL PROP, RR VEH, and RR PROP. Two groups (CTL) were not restrained prior to day 5, but received daily intra-BLA injections of vehicle (CTL VEH) or the β−AR antagonist propranolol (CTL PROP) on days 1-4. These groups provided a measure of mRNA expression in acutely stressed animals. The remaining two groups (RR) received daily 30 min restraint on days

1-4 which were followed by intra-BLA injections. One of these groups received daily restraint followed immediately by intra-BLA vehicle (RR VEH), which allowed us to measure changes in mRNA expression induced by repeated restraint. The RR PROP group received daily restraint followed by intra-BLA propranolol. This group provided a measure of whether β-AR blockade in the BLA would prevent changes in mRNA that were seen in the RR VEH group. On day 5, all animals were restrained for 30 minutes and sacrificed at the end of restraint, and brains were taken for in situ hybridization. The 30 minute timepoint for sacrifice was selected to allow for the measurement of c-fos mRNA, which is heavily expressed at this timepoint (Girotti, Pace, Gaylord, Rubin, Herman, & Spencer, 2006; Weinberg, Bhatt, Girotti, Masini, Day, Campeau, & Spencer, 2008), though as of this time I have not had the opportunity to examine c-fos. AVP and CRF mRNA in the PVN are not increased at 30 minutes into restraint (Ma & Lightman, 1998). At the time that this experiment was designed, I did not have reason to expect that BDNF mRNA would be altered 30 minutes into restraint. However, since designing the experiment evidence has come to light indicating that BDNF mRNA levels in the hippocampus are rapidly altered by acute restraint (Marmigere, Givalois, Rage, Arancibia, & Tapia-Arancibia, 2003), which I will address further in the discussion.

Animals were eliminated from experimental groups for a number of reasons, including misplaced cannulae, poor quality tissue sectioning for a given region, or unavailable sections for certain animals at the time a particular mRNA was hybridized. Thus, the starting n's for this experiment were: CTL VEH=10, CTL PROP=13, RR VEH=8, RR PROP=12. Final n's for AVP were: CTL VEH=6, CTL PROP=6, RR VEH=4, RR PROP=7. Final n's for CRF were: CTL VEH=7, CTL PROP=6, RR VEH=4, RR PROP=6. Final n's for BDNF were CTL VEH=8, CTL PROP=7, RR VEH=6, RR PROP=7.

Repeated restraint stress

The repeated restraint paradigm followed that described in Chapter 2. Repeatedly restrained animals were restrained for 30 minutes each day on days 1-4, after which animals were given intra-BLA injections of the β−AR antagonist propranolol. The dose and injection volume was the same as that used in the experiments in Chapter 2. On day

5, all animals were restrained for 30 minutes and sacrificed immediately after restraint to collect brains for in situ hybridization analysis.

Histology

At the end of 30 min restraint on day 5, all animals were immediately sacrificed and the brains rapidly removed and flash frozen in isopentane at -40˚C on dry ice. Brains were cryostat sliced in 14um sections on Superfrost Plus slides in 1 in 6 series, and stored at -80˚C until hybridization. Selected slides were cresyl violet stained for anatomical identification and confirmation of BLA cannulae placement. As in Chapter 2, animals were removed from analyses if cannulae were not directed at the BLA.

In situ hybridization

On the day of hybridization, slides were removed from the freezer and immediately postfixed in 4% paraformaldehyde for one hour. Following this they were washed 3 times in 2x sodium chloride-sodium citrate (SSC) buffer, acetylated in 0.1M triethanolamine containing 0.25% acetic anhydride for 10 minutes, washed for 5 minutes in 2x SSC, and finally dehydrated in progressive ethanol baths. Meanwhile, antisense and sense RNA probes were generated from cut cDNA (AVP and CRF cDNA graciously donated by Dr. Audrey Seashultz, BDNF cDNA graciously donated by Dr. Stanley Watson) and generated using ^{35}S labeled CTP and UTP, unlabeled ATP and GTP, and the appropriate polymerase. The probes were then purified using Tris Micro Bio-Spin Columns (BioRad) and the activities of the probes were measured using a β scintillation counter. Probes were considered successfully labeled if 1ul generated 1.5 million counts per minute (CPM) or more. Probes were then dissolved in hybridization buffer containing 50% formamide, 3x SSC, 1x Denhardt's solution, 10% Sodium Phosphate buffer pH 7.4, 10% dextran sulfate, 2% tRNA, and 10mM dithiothreitol. Probes were added to the hybridization buffer to a final concentration of 2,000,000 CPM/80ul. 80ul of antisense-containing hybridization buffer (for labeling the mRNA of interest) or sense-containing hybridization buffer (which labels nonspecifically) was applied to coverslips, which were then placed on each slide. Coverslipped slides in a container lined with filter paper soaked with 50% formamide. One slide per container was treated with

hybridization buffer containing the sense probe as a control. Containers were sealed to be as airtight as possible and incubated in a 55˚C oven for 16 hours.

At the end of the incubation, coverslips were removed by dipping the slides in 2x SSC. Slides were washed 3 times in 2x SSC, then incubated for 1 hour in 200µg/ml RNase A solution at 37˚C. Slides were then rinsed in decreasing concentrations of SSC (2x, 1x, 0.5x) followed by 1 hour incubation in 0.1x SSC at 65˚C. Slides were rinsed quickly in water and finally dehydrated in progressive ethanol baths. Radiolabeled slides were exposed to autoradiography film (Kodak) for varying lengths of time, depending on the probe (AVP, 3 and 4 hours; CRF, 64 and 72 hours, BDNF, 14 and 21 days). Autoradiographs were developed under darkroom conditions.

Statistical analysis

The developed autoradiographs were scanned at 1200 dpi and the optical density of brain regions on the films were measured using ImageJ software (NIH), such that more heavily labeled regions registered as higher values. The integrated density of each region of interest was expressed as a percentage of the integrated density of a nearby, unlabeled area of the same dimensions, using ImageJ software (NIH, Bethesda MD). The integrated density was coded by an observer blind to the treatment condition of each animal. Cresyl violet slides were examined after integrated density collection to ensure that data was being collected from approximately the same anatomical level in all animals and to remove PROP animals with misplaced cannulae. Percentage values of integrated density were analyzed in a 2x2 ANOVA for restraint condition (CTL, RR) x drug (VEH, PROP), followed when appropriate by Fisher's posthocs.

3.3 Results

AVP and CRF mRNA in the PVN

AVP mRNA density in the PVN can be seen in Figure 3.1, and CRF mRNA density in the PVN can be seen in Figure 3.2. AVP mRNA density was altered by restraint and propranolol administration. There was a significant Interaction between Stress and Drug condition ($F(1,19)=5.4$, $p \leq 0.05$) on AVP mRNA expression. Posthoc tests indicated that RR VEH animals had significantly increased AVP mRNA in the mpPVN compared to

CTL VEH ($p \leq 0.01$) and RR PROP ($p \leq 0.01$). There were no effects of Stress or Drug administration on CRF mRNA density in the PVN.

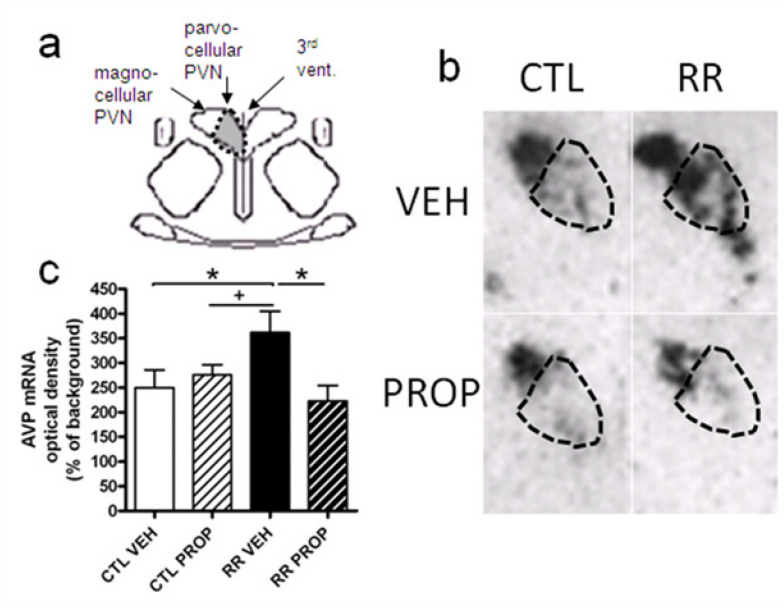

Figure 3.1 AVP mRNA in the PVN after repeated restraint and intra-BLA propranolol. In (a) is a depiction of the PVN and surrouding structures, and the medial parvocellular PVN, where AVP and CRF was measured, is indicated. In (b) is example PVN from the groups in this study, showing increased AVP mRNA density in the PVN of repeatedly restrained animals that received vehicle, but not propranolol, after daily restraint. In (c) is the graph of AVP mRNA density. RR VEH animals showed significant elevations (*) in AVP compared to CTL VEH and RR PROP, and tended (+, $p \leq 0.12$) to show increased AVP mRNA density compared to CTL PROP. (c) depicts means ± SEM.

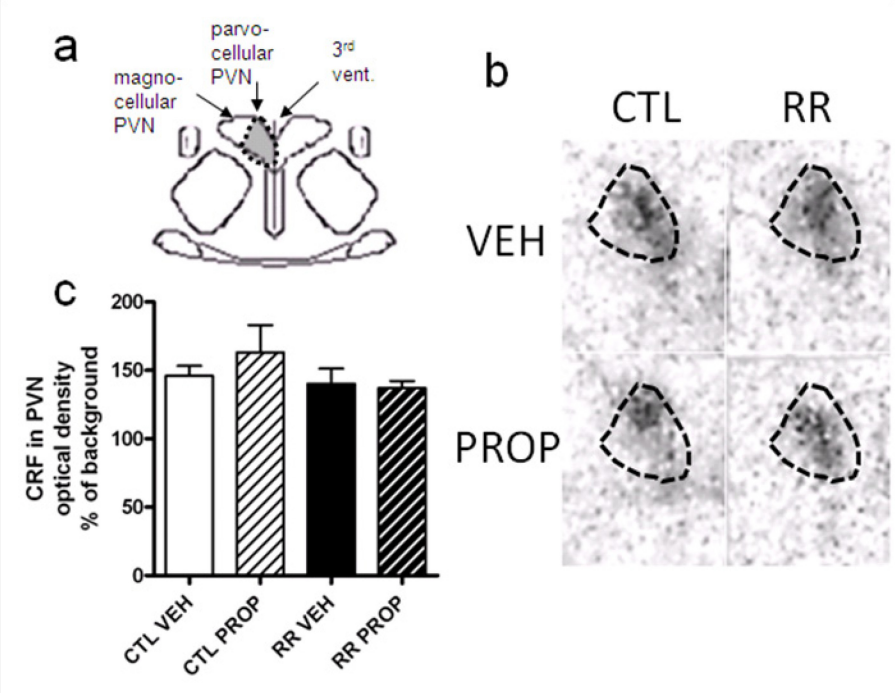

Figure 3.2 CRF mRNA in the PVN after repeated restraint and intra-BLA propranolol. In (a) is a depiction of the PVN and surrounding structures as in Figure 3.1(a). In (b) is an example of CRF mRNA density in each group. In (c) is a graph of CRF mRNA density in this experiment. No significant effects were seen in this measure. Figure depicts mean ± SEM.

BDNF mRNA in the BLA

BDNF mRNA density can be seen in Figure 3.3. During data collection from autoradiographs, I observed that BDNF expression in the BLA was not uniform. Rather, BDNF mRNA signal was much stronger in the more ventral "basal nucleus", and much less strong in the dorsal "lateral nucleus". For this reason, I analyzed the lateral and basal nuclei comprising the BLA separately. In the basal nucleus there was a significant Main Effect of Stress ($F(1,24)=7.3$, $p\leq0.05$) and a significant Interaction ($F(1,24)=5.8$, $p\leq0.05$). Posthoc tests indicate that BDNF mRNA was elevated in the basal nucleus of the BLA in RR VEH animals compared to all other groups (CTL VEH $p\leq0.01$; CTL PROP $p\leq0.05$: RR PROP $p\leq0.01$) (Figure 5). Neither repeated restraint nor β−AR blockade in the BLA affected BDNF mRNA in the lateral nucleus.

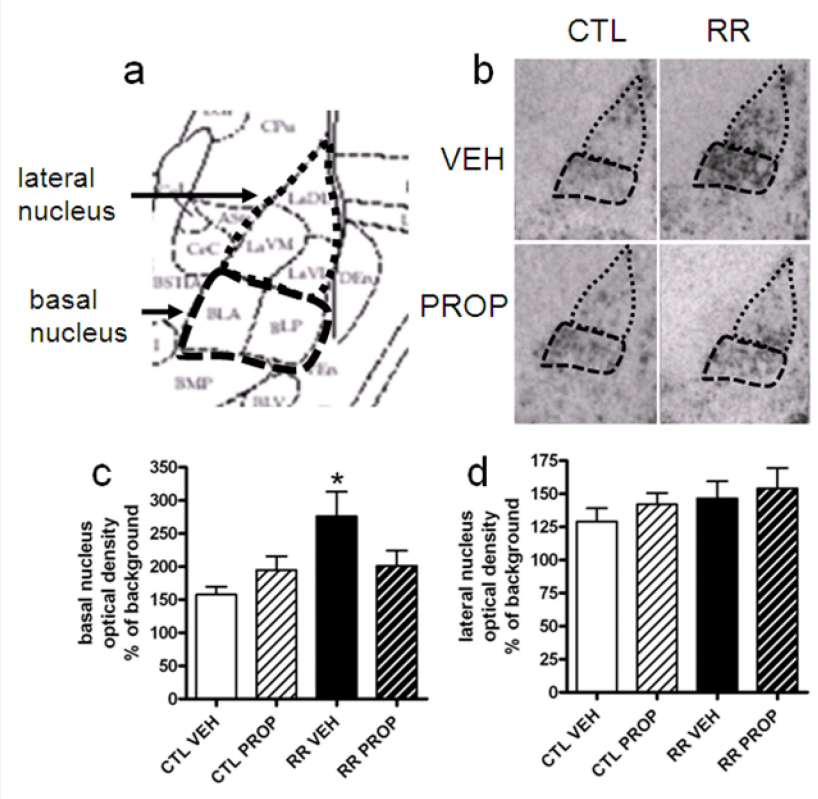

Figure 3.3 BDNF mRNA in the BLA after repeated restraint and intra-BLA propranolol. In (a) is a depiction of the BLA with the lateral and basal nuclei delineated. In (b) are example BLA from the groups in this study, depicting the increased BDNF mRNA in the basal nucleus of the BLA of repeatedly restrained rats given post-restraint vehicle, but not propranolol. In (c) and (d) are graphs of BDNF mRNA density in the basal (c) and lateral (d) nuclei. BDNF mRNA in the basal nucleus of RR VEH animals is significantly (*) higher than in all other groups. No differences were seen in BDNF mRNA density in the lateral nucleus. Graphs are means ± SEM.

BDNF mRNA in the hippocampus

BDNF mRNA in the hippocampus can be seen in Figure 3.4. Because BDNF expression in the hippocampus is strongest in the dentate gyrus than in other regions, the hippocampus is frequently divided into subregions for detailed analysis (Marmigere, Givalois, Rage, Arancibia, & Tapia-Arancibia, 2003) and I followed this approach. I found significant results in CA3 and the dentate gyrus. In CA3, there was a significant Main Effect of Stress ($F(1,22)=5.3$, $p\leq0.05$) and posthoc tests indicated that repeated restraint increased BDNF mRNA, regardless of drug treatment. In the dentate gyrus, there was a Main Effect of Stress ($F(1,22)=7.9$, $p\leq0.01$) and a significant Interaction between Stress and Drug ($F(1,22)=9.5$, $p\leq0.01$). Posthoc tests of this interaction

indicated that compared to CTL VEH, BDNF mRNA was elevated in CTL PROP ($p \leq 0.01$), RR VEH ($p \leq 0.005$), and RR PROP ($p \leq 0.01$).

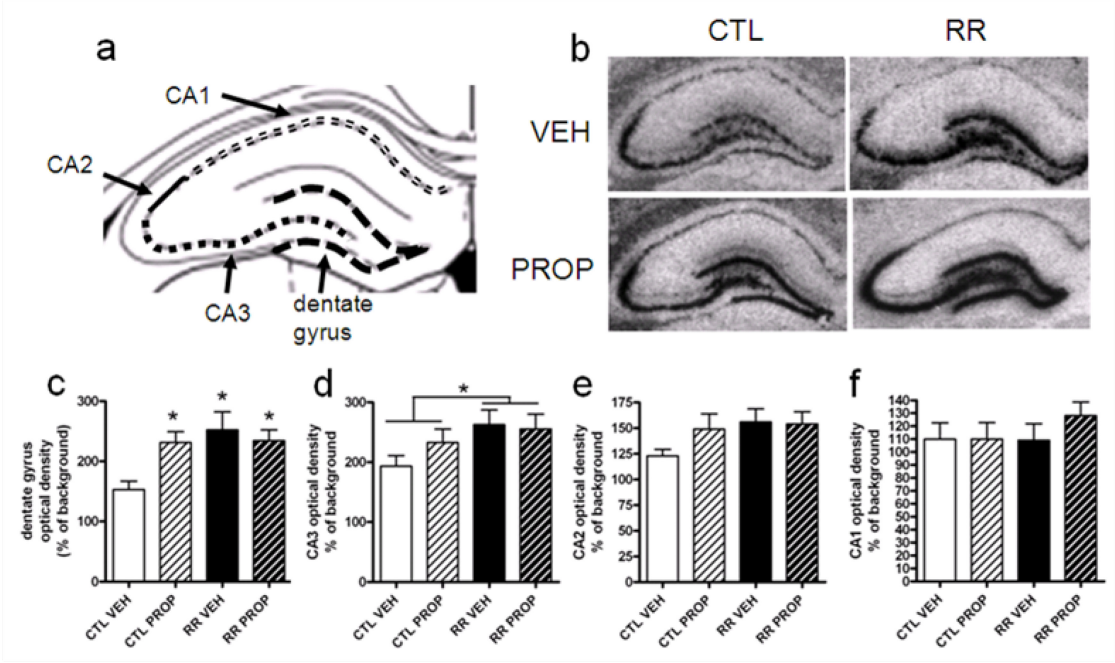

Figure 3.4 BDNF mRNA in the hippocampus after repeated restraint and intra-BLA propranolol. In (a) is a depiction of the hippocampus with the areas of interest demarcated. In (b) are representative hippocampi from groups in this experiment. In (c), (d), (e), and (f) are graphs of mean BDNF mRNA density in the dentate gyrus (c), CA3 (d), CA2 (e), and CA1 (f). In (c), BDNF mRNA in the dentate gyrus of CTL PROP, RR VEH, and RR PROP animals was significantly (*) elevated compared to CTL VEH. In (d), BDNF mRNA was significantly elevated in repeatedly stressed groups relative to controls. There were no effects in CA2 or CA1. Graphs are means ±SEM.

3.4 Discussion

The current experiment examined the effect of daily, post-restraint administration of the β−AR antagonist propranolol into the BLA on changes in mRNA in several brain regions that have been reported to occur after repeated stressors. I examined neuropeptide mRNA in the PVN as well as neurotrophin mRNA in the BLA and hippocampus. The PVN is the "motor" output region of the HPA axis (Sawchenko, Brown, Chan, Ericsson, Li, Roland, & Kovacs, 1996) and changes in neuropeptide mRNA in the PVN reflect changes in the drive to this area from other stress-response-regulatory brain regions (Lang & Davis, 2006; Ma & Morilak, 2005; Sawchenko, Brown, Chan, Ericsson, Li, Roland, & Kovacs, 1996) The neurotrophin BDNF has previously been shown to be decreased by repeated stressors and increased by conditioning and emotional processing

(Balu, Hoshaw, Malberg, Rosenzweig-Lipson, Schechter, & Lucki, 2008; Bergström, Jayatissa, Mørk, & Wiborg, 2008; Castillo, Figueroa-Guzman, & Escobar, 2006; Govindarajan, Rao, Nair, Trinh, Mawjee, Tonegawa, & Chattarji, 2006; Jones, Stanek-Rattiner, Davis, & Ressler, 2007; Monfils, Cowansage, & LeDoux, 2007; Ou & Gean, 2007; Rattiner, Davis, & Ressler, 2005).

First, those animals who received daily restraint with intra-BLA vehicle showed increased AVP mRNA in the PVN, a well-established effect of repeated stressors (Aubry, Bartanusz, Jezova, Belin, & Kiss, 1999; Ma & Lightman, 1998; Ma, Levy, & Lightman, 1997; Pinnock & Herbert, 2001). Intra-BLA administration of the β–AR antagonist propranolol after daily restraint prevented the increase of AVP at the mpPVN. Importantly, the BLA does not directly impinge on the PVN (Lang & Davis, 2006). Therefore, the mechanism of this increase in AVP mRNA must rely on alterations in plasticity between the PVN and its connecting structures that are modified by BLA neuronal signaling. At minimum, this could involve the BNST, which makes connections to the BLA and the PVN, and subsequent experiments could examine changes in BNST function and gene expression caused by repeated restraint and intra-BLA β-AR blockade. An additional implication of this finding is that the experience of repeated restraint and concomitant increase in HPA activity is not sufficient to induce changes in AVP mRNA expression in the PVN. The repeatedly restrained animals given propranolol after daily restraint still experienced restraint and daily elevations in HPA activity without altering AVP expression in the PVN.

No effects were seen of any manipulation on CRF mRNA in the PVN. This finding confirms that the effect of repeated restraint on neuropeptide activity in the PVN is limited to AVP. The lack of effect of repeated restraint or drug administration in the current experiment on CRF mRNA in the PVN is not unexpected. Repeated stressors such as restraint lead to increased AVP in CRF-expressing cells in the PVN (Aubry, Bartanusz, Jezova, Belin, & Kiss, 1999; De Goeij, Jezova, & Tilders, 1992), indicating that AVP and not CRF undergoes plastic changes as a result of prior stressor exposure. AVP in the PVN is thought to potentiate the effects of CRF and maintain HPA axis responsiveness to novel stressors in the face of prior repeated stressors and the

accompanying habituation and increased glucocorticoid negative feedback (Aguilera, Subburaju, Young, & Chen, 2008; Ma & Lightman, 1998).

In contrast to the expected effects of repeated restraint and propranolol on neuropeptide gene expression in the PVN, BDNF gene expression in the BLA and hippocampus was altered in an unexpected manner. In general, repeated restraint lead to an increase in BDNF mRNA expression in the BLA and some subregions of the hippocampus as seen at the end of restraint on day 5. As hypothesized, intra-BLA β-AR blockade after daily restraint prevented the restraint-induced increase BDNF mRNA in the BLA. However, intra-BLA β-AR blockade alone, repeated restraint alone, and a combination of these treatments all increased BDNF mRNA in the dentate gyrus. In the CA3, repeated restraint increased BDNF mRNA regardless of drug treatment. I will first address why BDNF mRNA might have been increased in repeatedly restrained animals in the current experiment, followed by a discussion of these effects in the BLA, then in the hippocampus.

Previous literature on the effects of stressor exposure on BDNF expression in the hippocampus and/or BLA has found that prior stressor exposure in the form of repeated restraint, social defeat, or chronic variable stress either decreases BDNF mRNA expression (Bergström, Jayatissa, Mørk, & Wiborg, 2008; Pizarro, Lumley, Medina, Robison, Chang, Alagappan, Bah, Dawood, Shah, Mark, Kendall, Smith, Saviolakis, & Meyerhoff, 2004; Tsankova, Berton, Renthal, Kumar, Neve, & Nestler, 2006) or has no effect (Allaman, Papp, Kraftsik, Fiumelli, Magistretti, & Martin, 2008). However, it is noteworthy that the animals in the current experiment were sacrificed at the end of 30 minute restraint on day 5, whereas in the studies cited above, animals were sacrificed a minimum of 24 hours after the last stressor exposure. Two explanations for this discrepancy present themselves. First, five days of 30 minute restraint may be a less severe stressor than those that have been previously studied, or is different in some other qualitative or quantitative fashion, that results in an overall increase in BDNF mRNA. Second, there may be a timecourse of BDNF mRNA expression changes induced by stressor exposure, wherein BDNF mRNA is rapidly increased during stressor exposure but tonic BDNF mRNA 24 hours later is reduced. There is evidence to support the idea

that the stressors used in the cited studies are more stressful than restraint. However, BDNF mRNA in the BLA has been found to be increased 2 hours after training by pairing odor with shock but not shock alone (Jones, Stanek-Rattiner, Davis, & Ressler, 2007). Shock is likely a more severe stressor than restraint (Bassett, Cairncross, & King, 1973; Hennessy, Levin, & Levine, 1977) but under the paired conditioned in Jones et al. lead to an increase in BDNF mRNA expression. Thus, differences in stressor severity probably do not contribute to the discrepancy in BDNF mRNA between these experiments. Indeed, the results of Jones et al. suggests that when BDNF mRNA is measured in relation to stimulus onset may play a role in whether increases or decreases are observed. There is evidence that the response of BDNF mRNA in the hippocampus to acute restraint is dynamic (Marmigere, Givalois, Rage, Arancibia, & Tapia-Arancibia, 2003). The timecourse identified significant elevations in BDNF mRNA expression by 15 and 30 minutes after restraint onset (Marmigere, Givalois, Rage, Arancibia, & Tapia-Arancibia, 2003)which have returned to baseline levels by 60 minutes (Bergström, Jayatissa, Mørk, & Wiborg, 2008; Marmigere, Givalois, Rage, Arancibia, & Tapia-Arancibia, 2003) after which levels of BDNF mRNA expression continue to decline (Marmigere, Givalois, Rage, Arancibia, & Tapia-Arancibia, 2003). I am unaware of whether a similar timecourse has been identified with regards to BDNF mRNA in the BLA induced by restraint specifically. However, in light of this literature, it is parsimonious to assume that the increased BDNF mRNA expression in the BLA and hippocampus in the current experiments reflects a dynamic effect induced by the 30 minutes of restraint on day 5. However, BDNF mRNA in each region was differentially regulated by prior restraint and intra-BLA drug administration, so the dynamic change on day 5 was influenced by these prior treatments.

BDNF mRNA in the BLA was not affected in the lateral nucleus, but was increased in the basal nucleus by repeated restraint. However, this increase was blocked by post-restraint intra-BLA propranolol. This result indicates that daily post-restraint activation of β-AR is important to allowing increases in BDNF expression in the basal nucleus of the BLA. Because the change in BDNF mRNA expression in the BLA parallels the effects of repeated restraint and intra-BLA propranolol on HPA activity and struggling behavior seen in Chapter 2, this finding suggests that increased BDNF in the BLA could

be one mechanism underlying the process of habituation to repeated stressors. This could be tested by examining of BDNF protein changes in the BLA after repeated restraint, and determining whether they parallel the changes in mRNA seen here. It might also be interesting to examine the effect of intra-BLA infusions of BDNF to determine if this enhances habituation.

BDNF mRNA in the hippocampus did not parallel HPA activity and struggling behavior from Chapter 2, but was altered in certain subregions by repeated restraint and/or intra-BLA β-AR blockade. BDNF mRNA was increased in repeatedly restrained animals in CA3 of the hippocampus, but was not altered by β-AR blockade in the BLA in either CTL or RR groups. It seems then that repeated restraint potentiates BDNF mRNA at the end of 30-minute restraint in CA3. This effect was slightly different in the dentate gyrus, in that BDNF mRNA was increased in animals that had received prior repeated restraint with and without post-restraint intra-BLA β-AR blockade, and in animals that had received β-AR blockade alone, without concurrent restraint. Repeated restraint may potentiate BDNF mRNA in this region as well, but this does not explain the increase in control propranolol animals undergoing their first restraint. The increase of hippocampal BDNF mRNA in acutely restrained animals has been suggested to reflect increased hippocampal plasticity in these animals induced by acute stressor exposure (see (Marmigere, Givalois, Rage, Arancibia, & Tapia-Arancibia, 2003). Such an argument would suggest that intra-BLA β-AR blockade increased the salience of the acute restraint in control animals, increasing the plasticity occurring in the hippocampus as a response. However, regardless of the cause of these effects, these results suggest that BDNF mRNA in the hippocampus at the end of 30 minute restraint is not directly relevant to habituation to the repeated stressor, as it does not parallel habituation, though it may be involved in stress response regulation in some other way. It also suggests that intra-BLA β-AR blockade alone, which did not induce significant changes in HPA activity or struggling behavior in Chapter 2, nevertheless exerts changes in neural function. This may be of interest in future investigations.

What might be the role of increased BDNF in repeatedly stressed animals in both the BLA and hippocampus? It has been suggested that the rapid increase in BDNF mRNA

expression by acute restraint in the hippocampus may be involved with some aspect of learning about the restraint (Marmigere, Givalois, Rage, Arancibia, & Tapia-Arancibia, 2003). This is in concordance with the literature examining the role of BDNF on learning and memory in the hippocampus and BLA, in which BDNF infusions have been found to exert acute actions which enhance the acquisition of learned responses (Castillo, Figueroa-Guzman, & Escobar, 2006; Jones, Stanek-Rattiner, Davis, & Ressler, 2007; Lee, Everitt, & Thomas, 2004; Moguel-Gonzalez, Gomez-Palacio-Schjetnan, & Escobar, 2008). It is noteworthy that these effects are in aversive learning paradigms, specifically conditioned taste aversion (Castillo, Figueroa-Guzman, & Escobar, 2006; Moguel-Gonzalez, Gomez-Palacio-Schjetnan, & Escobar, 2008), olfactory fear conditioning (Jones, Stanek-Rattiner, Davis, & Ressler, 2007), and contextual fear conditioning (Lee, Everitt, & Thomas, 2004). Aversive experiences may temporarily increase BDNF expression in the hippocampus and BLA to enhance the recall of the experience, and this recall may be important to influencing subsequent stress responses.

The overall results of this chapter indicate that intra-BLA β−AR blockade can exert effects within and beyond the BLA, altering gene expression at the PVN, BLA, and hippocampus. The increases in AVP mRNA in the PVN and BDNF mRNA in the BLA parallel the effects of post-restraint β−AR blockade on HPA activity and behavior, and thus may be significantly involved in habituation to repeated stressors. These peptide and trophic responses reflect the brain's dynamic response to the surge of activity elicited by prior stressors, and thus paint an interesting and complex picture of what processes underlie stress response habituation. Furthermore, given the wide variety of paradigms for which memory consolidation can be altered by intra-BLA β−AR manipulations, these results suggest that BDNF in the BLA in particular may be crucially involved in β-AR effects on memory consolidation. However, little is known of the effects β−AR blockade has within the BLA at cellular levels that alters its subsequent plasticity and activity in other stress-response-regulatory brain regions. In the next chapter, I explore changes in BLA intracellular activity induced by repeated restraint and intra-BLA β−AR blockade that may begin to shed light on these effects.

Chapter 4.

Intracellular signaling in the BLA regulates HPA activity to restraint

4.1 Introduction

In the previous chapters, I found that post-restraint β-AR signaling in the BLA exerts a profound influence on neural and behavioral changes induced by repeated restraint. Blocking β-AR after daily restraint prevented the habituation of ACTH activity and struggling behavior to repeated restraint in Chapter 2. In Chapter 3, this manipulation prevented repeated restraint-induced increases in AVP mRNA in the PVN and BDNF mRNA in the BLA. Together, these effects indicate that normally β-AR activation in the BLA of an animal exposed to a repeated stressor induces changes in the function of BLA neurons. Signals from these neurons then alter the activity of downstream brain regions that regulate HPA and behavioral responses to stressors. This raises the question of whether intracellular signaling downstream from activated β−AR are altered in the BLA of repeatedly restrained animals, whether blocking β−AR prevents activation of these intracellular mechanisms, and whether this activation is functionally relevant to the habituation of HPA activity.

β-AR are among the most heavily studied G-protein coupled receptors. A simplified diagram of the intracellular signals activated by β-AR that will be discussed in this chapter is shown in Figure 4.1. Classically, they are coupled to Gs, so that activation of the receptor activates cyclic adenosine monophosphate (cAMP) which phosphorylates protein kinase A (PKA) (Roozendaal, Schelling, & McGaugh, 2008; Tronson, Wiseman, Olausson, & Taylor, 2006; Zheng, Shen, Xiong, Yang, & He, 2008). cAMP activation is a necessary step for β-AR effects in the BLA on modulating memory consolidation. Intra-BLA infusions of the cAMP analog 8-Br-cAMP either before or after single-trial inhibitory avoidance training enhances the consolidation of memory for that training (Roozendaal, Quirarte, & McGaugh, 2002; Roozendaal, Schelling, & McGaugh, 2008).

Blocking cAMP activation in the BLA with the inhibitor Rp-cAMPs either before or after training blocks the consolidation of memory for that training (Roozendaal, Quirarte, & McGaugh, 2002; Roozendaal, Schelling, & McGaugh, 2008), and blocks the enhancement of memory consolidation by β−AR agonist (Ferry, Roozendaal, & McGaugh, 1999). Blocking PKA activation can also inhibit the reconsolidation of Pavlovian fear conditioning (Tronson, Wiseman, Olausson, & Taylor, 2006).

There is increasing evidence that β-AR can also activate extracellular signal-regulated kinase (ERK, also known as mitogen activated protein kinase or MAPK) pathways in the cell, and that ERK is important in BLA-mediated learning and memory. ERK phosphorylation in the BLA is increased by Pavlovian fear conditioning (Schafe, Atkins, Swank, Bauer, Sweatt, & Ledoux, 2000). Blocking ERK phosphorylation via inhibition of MEK (MAPK/ERK kinase) blocks the consolidation of both Pavlovian fear conditioning (Schafe, Atkins, Swank, Bauer, Sweatt, & Ledoux, 2000) and the consolidation of operant avoidance conditioning (Quevedo, Vianna, Roesler, Martins, de-Paris, Medina, & Izquierdo, 2005; Rossato, Bonini, Coitinho, Vianna, Medina, Cammarota, & Izquierdo, 2004). This demonstrates that ERK phosphorylation in the BLA is important to BLA-mediated aversive learning. β−AR phosphorylation of ERK can occur via 1) direct activation of MEK by activated PKA, or 2) changed β-AR G-protein coupling by activated PKA that leads to direct β-AR activation of MEK and therefore ERK (Waltereit & Weller, 2003; Zheng, Shen, Xiong, Yang, & He, 2008; Zou, Komuro, Yamazaki, Kudoh, Uozumi, Kadowaki, & Yazaki, 1999). Together, these findings suggest that one consequence of β−AR activation in the BLA of repeatedly stressed animals may be ERK phosphorylation. ERK phosphorylation in the BLA may be of particular importance in the acquisition of experience-dependent changes in HPA activity and behavior to a homotypic stressor.

Downstream of the phosphorylation of both PKA (Roberson, English, Adams, Selcher, Kondratick, & Sweatt, 1999; Waltereit & Weller, 2003) and, according to some reports, ERK (Chwang, Arthur, Schumacher, & Sweatt, 2007; Roberson, English, Adams, Selcher, Kondratick, & Sweatt, 1999; Waltereit & Weller, 2003), is the activation of cAMP response element binding protein (CREB). CREB plays a direct role in gene

transcription as a transcription factor, and plays an indirect role in facilitating gene transcription via its involvement in chromatin remodeling. Chromatin remodeling has been an area of increasing focus with regards to stressors and emotional stimuli (Jiang, Langley, Lubin, Renthal, Wood, Yasui, Kumar, Nestler, Akbarian, & Beckel-Mitchener, 2008; Renthal, Maze, Krishnan, Covington, Xiao, Kumar, Russo, Graham, Tsankova, Kippin, Kerstetter, Neve, Haggarty, McKinsey, Bassel-Duby, Olson, & Nestler, 2007; Tsankova, Berton, Renthal, Kumar, Neve, & Nestler, 2006) and learning and memory for aversive events (Chwang, Arthur, Schumacher, & Sweatt, 2007; Oliveira, Wood, McDonough, & Abel, 2007; Roberson, English, Adams, Selcher, Kondratick, & Sweatt, 1999). CREB, in conjunction with other effector proteins, is able to modify histone proteins, which form the bulk of the chromatin and around which DNA is wound. CREB and its effector proteins increase histone acetylation, a specific modification that decreases the affinity of the histone protein for DNA. The released DNA is thus more accessible to transcription factors, allowing increased gene transcription (Jiang, Langley, Lubin, Renthal, Wood, Yasui, Kumar, Nestler, Akbarian, & Beckel-Mitchener, 2008). Increased acetylation of histone H3 (AcH3) in CA1 of the hippocampus is necessary for the consolidation of contextual fear conditioning (Miller, Campbell, & Sweatt, 2008). Increased phosphorylation-acetylation of histone H3 in the dentate gyrus is associated with the increase in immobility elicited by a second exposure to forced swimming (Chandramohan, Droste, Arthur, & Reul, 2008). Given the importance of cAMP and potentially ERK signaling mediated by β−AR activated by repeated restraint, and given that both factors could potentially modify histone acetylation via CREB, there may be changes in histone acetylation in the BLA of repeatedly restrained animals. This could be one mechanism by which BLA β-AR activation changes HPA activity and behavior to a homotypic stressor.

In the current experiments, I looked at specific intracellular mechanisms in the BLA to determine if they were changed by repeated restraint and/or post-stress β-AR blockade. In Experiment 4.1, I conducted a pilot experiment to examine the density of phosphorylated ERK (pERK) and acetylated histone H3 (AcH3) in the BLA and hippocampus of animals immediately after a first restraint, a fifth restraint, or no restraint.

These results indicated that acute restraint was sufficient to alter pERK and AcH3 compared to unrestrained animals, so in the subsequent experiment I examined basal differences rather than those seen at the end of restraint. In Experiment 4.2, I examined basal density of pERK and AcH3 in the BLA and hippocampus on day 5 of animals that had received 4 prior days of restraint followed each day by the β-AR antagonist propranolol as in Chapters 2 and 3. Finally, in Experiment 4.3 I infused the MEK inhibitor U0126 into the BLA after each of 4 days of restraint in a design similar to Chapters 2 and 3 to determine if pharmacological reduction in ERK phosphorylation in the BLA would have direct effects on HPA activity in response to restraint on day 5.

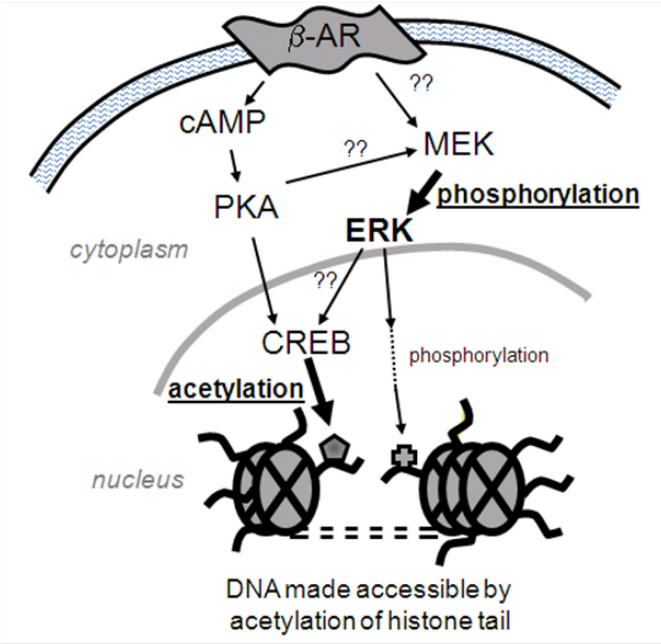

Figure 4.1 Diagram of intracellular signaling mechanisms discussed in Chapter 4. β-AR is classically thought to act via cAMP/PKA mechanisms, one important action of which is CREB phosphorylation, which leads to increased histone acetylation (AcH3). β-AR may also phosphorylate ERK (pERK), which may also activate CREB. pERK and AcH3, which were explicitly measured in this chapter, are emphasized. See text for further details.

4.2 Methods

Animals

Animals were sourced and housed as described in Chapter 2. All experiments were conducted in accordance with the Children's Hospital of Philadelphia IACUC guidelines.

Experimental Design

Experiment 4.1: Is pERK and/or AcH3 in the BLA and hippocampus activated by restraint?

This was a pilot experiment. It was designed to test the hypothesis that, compared to acutely restrained animals, repeatedly restrained animals would exhibit alterations in ERK phosphorylation (pERK) and histone H3 acetylation (AcH3). A subset of the animals used in this experiment were those used in the first experiment in Appendix A (Grissom, Kerr, & Bhatnagar 2008) to examine behavioral habituation to restraint. There were three groups in this study. One set of animals was unstressed (US) and sacrificed basally. A second set was restrained once for 30 minutes and sacrificed at the end of this acute restraint (CTL). The third set was restrained for 30 minutes once a day for 5 days, and were sacrificed at the end of the 5th restraint (RR).

Starting n's for Experiment 4.1, and final n's for BLA pERK only: US=6, CTL=16, RR=17. BLA AcH3: US=6, CTL=12, RR=13. Hippocampus pERK: US=6, CTL=9, RR=10. Hippocampus AcH3: US=6, CTL=9, RR=10. BLA AcH3 levels were not measured in those animals which had extremely low (<3 SD) levels of total H3, which indicated insufficient protein concentrations. Westerns for the hippocampal tissue were not performed on all samples in this experiment.

Experiment 4.2: Are changes in basal pERK and AcH3 in the BLA and hippocampus induced by repeated restraint prevented by intra-BLA administration of propranolol?

Having found in Experiment 4.1 that repeated restraint induced decreases in pERK and increases in AcH3, I examined whether pERK and AcH3 were prevented by intra-BLA administration of the β−AR antagonist propranolol as the habituation of HPA and behavioral responses to repeated restraint were prevented by propranolol. Based on the demonstration of rapid changes in pERK and AcH3 signaling as a result of 30 minute restraint when compared to unstressed animals in Experiment 4.1, I elected to sacrifice animals in Experiment 4.2 under basal conditions on day 5 to examine tonic changes induced by prior restraint and drug administration. The literature examining histone

modifications in response to stressors and learning generally examines AcH3 under basal conditions (Chandramohan, Droste, Arthur, & Reul, 2008; Miller, Campbell, & Sweatt, 2008; Renthal, Maze, Krishnan, Covington, Xiao, Kumar, Russo, Graham, Tsankova, Kippin, Kerstetter, Neve, Haggarty, McKinsey, Bassel-Duby, Olson, & Nestler, 2007). In the current experiment, I examined 4 groups, in a 2x2 design: CTL VEH, receiving no restraint on days 1-4 but daily intra-BLA vehicle injections; CTL PROP, receiving no stress d1-4 but daily intra-BLA PROP; RR VEH, receiving 30 min restraint d1-4 followed immediately by intra-BLA vehicle; and RR PROP, receiving 30 min restraint d1-4 followed immediately by intra-BLA PROP. All animals were sacrificed under nonstressed, basal conditions on day 5 between 0900-1200h.

Starting n's for Experiment 4.2 were: CTL VEH=12, CTL PROP=15, RR VEH=12, RR PROP=21. As in previous chapters, animals were eliminated based on misplaced cannulae, though this could not be judged as accurately. As in Experiment 4.1, BLA AcH3 was not analyzed for those samples containing extremely low levels of total histone H3. Final n's were: BLA pERK: CTL VEH=11, CTL PROP=11, RR VEH=10, RR PROP=11. BLA AcH3: CTL VEH=8, CTL PROP=10, RR VEH=10, RR PROP=8. Hippocampus pERK: CTL VEH=11, CTL PROP=11, RR VEH=10, RR PROP=11. Hippocampus AcH3: CTL VEH=11, CTL PROP=11, RR VEH=10, RR PROP=10.

Experiment 4.3: Does intra-BLA MEK inhibition enhance HPA habituation to repeated restraint?

In Experiment 4.2, animals that were repeatedly restrained for 4 days exhibited reductions in pERK in the BLA, and this reduction was not seen in repeatedly restrained animals given intra-BLA propranolol. I hypothesized that decreased ERK phosphorylation in the BLA seen in Experiment 4.2 could underlie HPA habituation. Thus, blocking the activity of MEK and therefore reducing ERK phosphorylation in the BLA would enhance HPA habituation to repeated restraint. The MEK inhibitor U0126 used in this experiment has previously been shown to block ERK phosphorylation in the BLA (Duvarci, Nader, & Ledoux, 2005; Schafe, Swank, Rodrigues, Debiec, & Doyere, 2008; Schafe, Atkins, Swank, Bauer, Sweatt, & Ledoux, 2000); further detail is below under "Drugs". A total of 4 groups were used in a 2x2 design similar to that used in

Experiment 4.2: CTL VEH and RR VEH animals were treated on days 1-4 as in Experiment 2 above; CTL MEKI animals received no restraint d1-4 but daily intra-BLA administration of U0126; RR MEKI animals received daily restraint on d1-4 followed immediately afterwards by intra-BLA U0126. The administration of the MEK inhibitor immediately after restraint was decided on based on literature demonstrating that this timing of administration should be effective (). On day 5, no drug was given and all groups were restrained for 30 minutes and had repeated blood samples taken at 0, 15, 30, and 60 minutes.

Starting n's for this experiment were: CTL VEH=16, CTL MEKI=13, RR VEH=14, CTL MEKI=22. Final n's for Experiment 4.3 were: 0 minute: CTL VEH=12, CTL MEKI=9, RR VEH=12, CTL MEKI=13. 15 minute: CTL VEH=11, CTL MEKI=9, RR VEH=10, CTL MEKI=12. 30 minute: CTL VEH=11, CTL MEKI=8, RR VEH=10, CTL MEKI=12. 60 minute: CTL VEH=12, CTL MEKI=8, RR VEH=12, CTL MEKI=13. Integrated ACTH: CTL VEH=11, CTL MEKI=8, RR VEH=10, CTL MEKI=12.

Surgery

In Experiments 4.2 and 4.3 animals received surgery to implant intra-BLA guide cannulae as described in Chapter 2.

Stress

The repeated restraint paradigm followed that described in Chapter 2.

Drugs

The β-AR antagonist propranolol (PROP; DL propranolol, Sigma) was dissolved in phosphate buffered saline (PBS) as described in Chapter 2. Injections of PROP and its vehicle in Experiment 4.2 were 0.2 ul/side. For Experiment 4.3, each day 1mg MEK inhibitor U0126 (MEKI; Promega) was dissolved in 234 ul DMSO per manufacturer instructions, which was mixed with 266 ul PBS to a final concentration of 1ug/0.5ul. Injections of MEKI and its vehicle were 0.5ul/side. This dilution protocol and injection volume is the same as previous literature examining the effect of MEK blockade in the BLA (Schafe, Atkins, Swank, Bauer, Sweatt, & Ledoux, 2000). The increased injection

volume for MEKI was necessary to obtain the appropriate amount of U0126 due to the required volume of solute. Injections were delivered over a minute and the injector cannulae were left in place an additional 15 seconds to allow for drug diffusion.

Tissue preparation

Brains were rapidly removed and a 2mm thick section from approximately -1.8 bregma to -3.8 bregma was removed using an ice-cold rat brain mold (Braintree Scientific). From this section, the bilateral BLA was punched out using a circular 1mm punch (Fine Science Tools). The BLA was localized as the area medial to the ventralmost extent of the external capsule. The dorsal hippocampus was free-dissected. Bilateral tissue samples for each animal were pooled in a microcentrifuge tube and immediately frozen on dry ice and stored at -80°C until further processing. On the day of homogenization, ice-cold lysis buffer including 10mM Tris Base, 5mM EDTA and1% HALT protease inhibitor cocktail (Thermo) and 0.25% Phosphosafe phosphatase inhibitor (Novagen) was added to each punch-containing tube and homogenized using a handheld motor-driven pestle (Kimble Kontes). Homogenate was spun at 14,000 rpm in an Eppendorf 5415C centrifuge at 4°C for 15 minutes. The supernatant was aliquoted as the cytoplasmic fraction. The pellet was used to acid-extract the nuclear proteins with 0.2M HCl + 10% glycerol, which were then precipitated with ice-cold acetone and centrifuged as above to collect the pellet, which was finally dissolved in 9M urea and stored as the nuclear fraction. Between 5-25ul of each sample, based on known approximate protein concentration, was removed to run a BCA protein concentration assay (ThermoFisher) and the remainder was frozen at -80°C until run in the Western blot. For the BLA, protein concentrations of the nuclear fraction were sometimes insufficient for analysis, resulting in reduced n for analysis of acetylated histone in this region,

Western Blotting

Protein concentrations for different sample preparations vastly differed. Because of this, the amount of protein loaded onto gels for SDS-PAGE for each sample type was determined by pilot testing to determine minimum required (for BLA AcH3 in nuclear samples) or optimal protein volumes for detection of the protein of interest. Nuclear

samples contained histones and were assayed for AcH3, while cytoplasmic fractions were assayed for pERK. Thus, BLA cytoplasmic samples (10 ug), BLA nuclear samples (2 ug), hippocampus cytoplasmic samples (25 ug), and nuclear samples (10 ug) were mixed with an equal volume of loading buffer containing 50mM β–mercaptoethanol and Laemmli buffer. These samples were loaded on 4-15% precast Tris-HCl polyacrylamide gel (Biorad) and electrophoresed for 35 minutes at 200V, after which they were transferred to Immobilon-FL PVDF membranes (Millipore) for 60 minutes at 100V. After transfer membranes were immediately blocked with 1:3 Odyssey blocking buffer (Licor) to TBS for 1 hour at room temperature, then washed 3 times for 10 minutes in TBS+0.1% Tween-20. Blots of the cytoplasmic fractions were then incubated overnight at 4°C with primary antibodies to total ERK 1/2 in rabbit (1:1000, Cell Signaling) and pERK 1/2 in mouse (1:1000, Santa Cruz). Each antibody recognized both ERK 1 and ERK 2, which are highly homologous (Selcher, Nekrasova, Paylor, Landreth, & Sweatt, 2001) but differ in molecular weight (ERK 1 is 44 kilodaltons, ERK 2 is 42 kilodaltons). Blots of the nuclear fractions were incubated overnight at 4°C with primary antibodies to total histone H3 in mouse (1:1000, Abcam) and acetylated (Lysine 9 and 14) histone H3 in rabbit (1:20,000, Upstate). The following morning the membranes were washed 3 times for 10 minutes in TBS+Tween, then incubated for 1 hour at room temperature with infrared fluorescence secondary antibodies (1:5000 donkey-anti-rabbit in 700nm red, and 1:5000 donkey-anti-mouse in 800nm green, both Licor) in 1:3 Odyssey blocking buffer to TBS+Tween. Membranes were immediately washed twice for 10 minutes in TBS+Tween and once in TBS. Membranes were then scanned using the Odyssey scanning system that allows separate visualization of the two secondary antibody colors. Optical density measurements of the total protein bands and the comparable phosphorylated or acetylated bands were obtained and converted into values for pERK 1/total ERK 1, pERK 2/ total ERK 2, or AcH3/total H3 for subsequent analysis. The two isoforms of ERK, ERK 1 and ERK 2, were analyzed separately as they have been shown to produce different results in some paradigms (Selcher, Nekrasova, Paylor, Landreth, & Sweatt, 2001; Shen, Tsimberg, Salvadore, & Meller, 2004).

Blood Sampling and Radioimmunoassays

For Experiment 4.3, blood samples were collected during restraint and measured for ACTH using kits from MP Biomedicals as described in Chapter 2. Corticosterone was not assayed.

Statistical analyses

All statistics were analyzed using Statview. Experiment 4.1 contained three groups, one which received no stressor (US), one which was sacrificed at the end of a first 30 minute restraint (CTL) and one which was sacrificed at the end of a 5^{th} 30 minute restraint (RR). Results were analyzed using one-way ANOVA, with Fisher's posthocs where appropriate.

Experiments 4.2 and 4.3 used similar 2x2 designs and as such were analyzed with 2x2 ANOVA for Stress (CTL, RR) by Drug (VEH, PROP) for Experiment 4.2, or (VEH, MEKI) for Experiment 4.3. Significant omnibus results were followed by Fisher's posthocs.

4.3 Results

Experiment 4.1: Are ERK phosphorylation and/or histone H3 acetylation in the BLA and hippocampus altered as a result of restraint?

The results of this experiment can be seen in Figure 4.2 for the BLA, and Figure 4.3 for

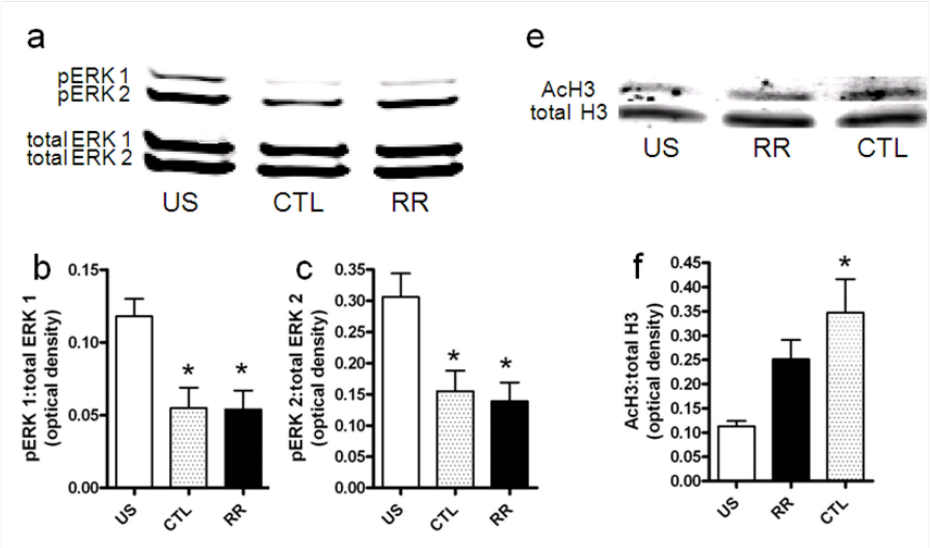

Figure 4.2 BLA pERK and AcH3 after no stress, a single restraint, or repeated restraint in Experiment 4.1. (a) is an example Western blot for pERK and total ERK in this experiment. (b) depicts pERK1/ERK1 and c depicts pERK2/ERK2. (e) is an example western blot for AcH3 and total H3 in this experiment. (f) depicts AcH3:H3. pERK1/2 in (b) and (c) were significantly (*) reduced in the BLA of both CTL and RR animals at the end of restraint as compared to a basal animal (US). AcH3 was significantly increased in the BLA of CTL animals at the end of a first 30 minute restraint as compared to basal US animals. RR animals at the end of a 5th restraint were not different from either US or CTL animals. Thus, acute stress reduces pERK and increases AcH3. Figures are mean ± SEM.

the hippocampus. In the BLA, pERK1/2 were significantly reduced at the end of 30 minute restraint in both CTL and RR groups compared to the basal US animals (ERK 1: $F(2,36)=3.7$, $p\leq0.05$; ERK 2: $F(2,36)=4.2$, $p\leq0.05$; CTL vs. US posthoc $p\leq0.01$; RR vs. US posthoc $p\leq0.01$). pERK in CTL and RR rats at the end of 30 minute restraint did not differ. AcH3 in the BLA was significantly altered by Stress ($F(2,28)=3.5$, $p\leq0.05$). Posthoc analysis indicated that AcH3 was increased in the BLA of CTL rats compared to US basal animals ($p\leq0.01$), but BLA AcH3 in RR rats was not different from either CTL or US animals. No differences in pERK or AcH3 were seen in the hippocampus.

Overall, I found that restraint decreased ERK phosphorylation and increased histone H3 acetylation in the BLA.

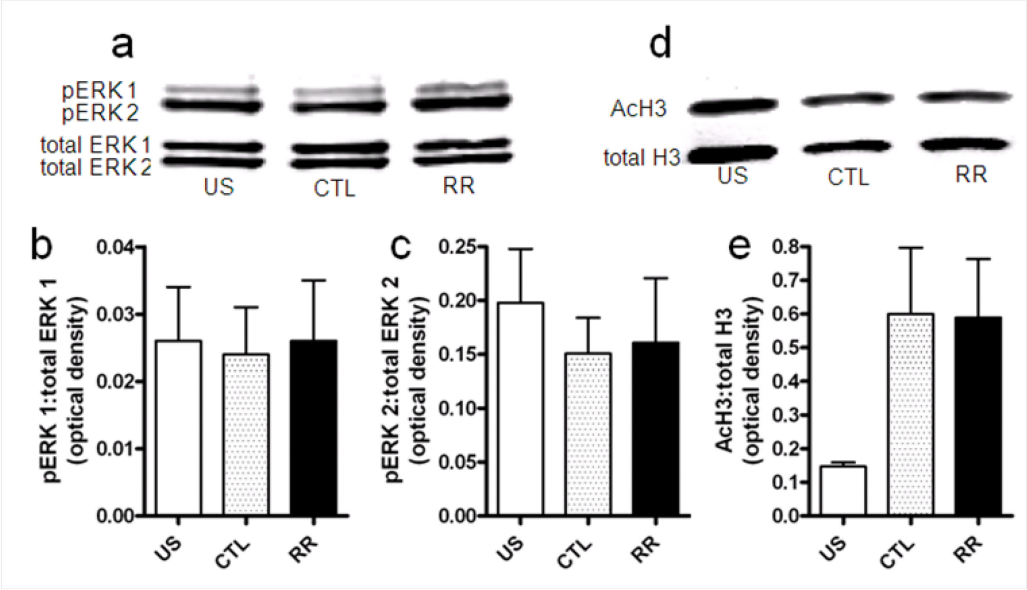

Figure 4.3 Hippocampus pERK and AcH3 after no stress, a single restraint, or repeated restraint in Experiment 4.1. (a) is a representative Western blot for pERK and total ERK in this experiment. (b) depicts pERK1/ERK1 and (c) depicts pERK2/ERK2. (e) is a representative Western blot for AcH3 and total H3 in this experiment. (f) depicts AcH3:H3. No significant differences were seen in pERK or AcH3 in the hippocampus. Figures depict means ± SEM.

Experiment 4.2: Are pERK and AcH3 changes induced in the BLA and hippocampus by repeated restraint prevented by intra-BLA administration of propranolol?

The results of this experiment are shown in Figures 4.4 for the BLA and Figure 4.5 for the hippocampus. pERK 1/2 in the BLA was significantly altered by repeated restraint and propranolol injection (pERK 1: Stress x Drug Interaction $F(1,39)=8.7$, $p \leq 0.005$; pERK 2: Stress x Drug Interaction $F(1,39)=4.4$, $p \leq 0.05$). Posthoc tests indicate that pERK 1/2 was significantly reduced in repeatedly restrained vehicle animals (RR VEH) compared to both control animals given vehicle (CTL VEH, $p \leq 0.01$) and repeatedly restrained animals given propranolol (RR PROP, $p \leq 0.05$), and tended to be reduced

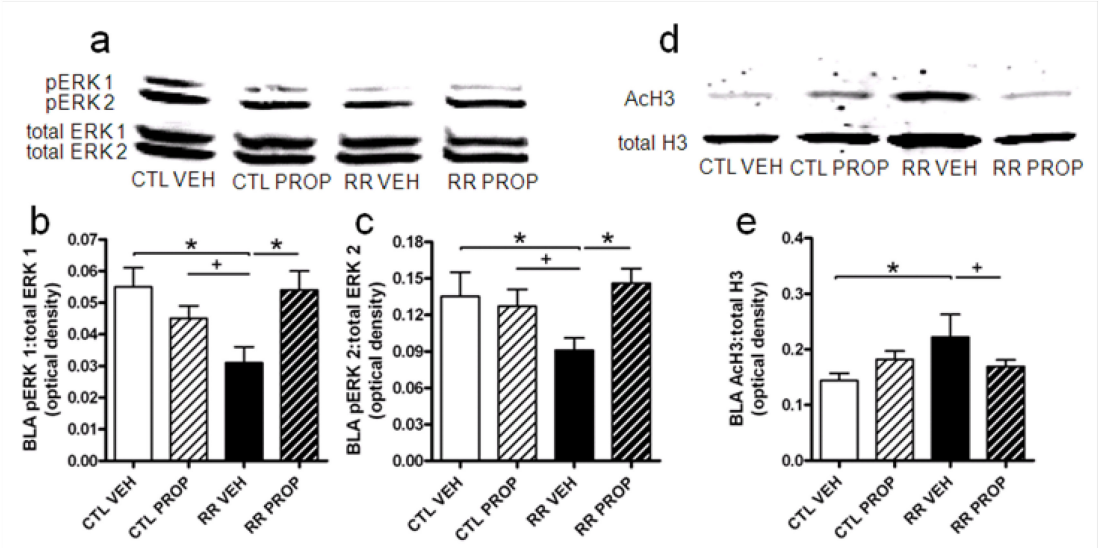

Figure 4.4 BLA pERK and AcH3 in animals sacrificed basally after prior repeated restraint and intra-BLA propranolol in Experiment 4.2. In this experiment, all animals were sacrificed basally on day 5. Thus all differences are tonic changes induced by prior restraint and intra-BLA drug administration. (a) is a representative Western blot for pERK and total ERK in this experiment. (b) depicts pERK1/ERK1 and (c) depicts pERK2/ERK2. (d) is a representative Western blot for AcH3 and total H3 in this experiment. (e) depicts AcH3:H3. pERK1/2 in (b) and (c) was significantly (*) reduced in the BLA of repeatedly restrained animals given intra-BLA vehicle after daily restraint (RR VEH) as compared to unstressed animals which received intra-BLA vehicle (CTL VEH) or repeatedly restrained animals given intra-BLA propranolol (RR PROP). The difference between RR VEH and CTL PROP tended towards significance (+, $p = 0.09$). AcH3 in (e) was significantly increased in the BLA of RR VEH animals as compared to CTL VEH, and tended ($p = 0.08$) to be increased compared to RR PROP. Thus, propranolol in the BLA after daily restraint attenuates intracellular signaling changes in the BLA induced by repeated restraint. See text for complete results. Figures depict means ± SEM.

compared to control animals given propranolol (CTL PROP p=0.09). AcH3 was increased in the BLA of RR VEH compared to CTL VEH (p≤0.05), and tended (p=0.08)

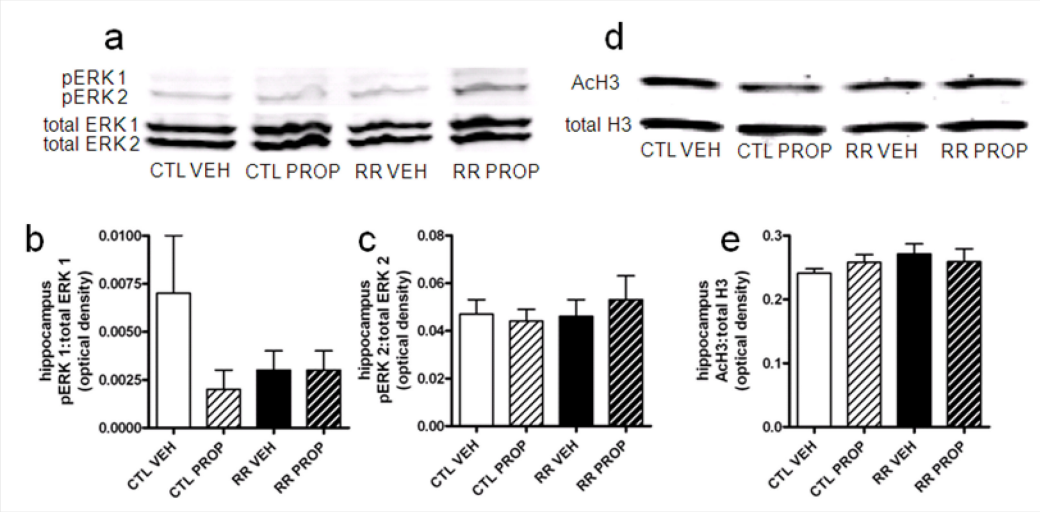

Figure 4.5 Hippocampus pERK and AcH3 in animals sacrificed basally after prior repeated restraint aind intra-BLA propranolol in Experiment 4.2. In this experiment, all animals were sacrificed basally on day 5. Thus all differences are tonic changes induced by prior restraint and intra-BLA drug administration. (a) is a representative Western blot for pERK and total ERK in this experiment. (b) depicts pERK1/ERK1 and (c) depicts pERK2/ERK2. (d) is a representative Western blot for AcH3 and total H3 in this experiment. (e) depicts AcH3:H3. No significant differences were seen in pERK or AcH3 in the hippocampus. Figures depict means ± SEM.

to be increased in RR VEH compared to RR PROP (Stress x Drug Interaction $F(1,33)=4.5$, $p\leq0.05$). No changes were seen in pERK or AcH3 in the hippocampus. Overall, tonic pERK was decreased, and AcH3 was increased, by repeated restraint, but propranolol in the BLA after daily restraint attenuated these changes.

Experiment 4.3: Does intra-BLA MEK inhibition enhance HPA habituation to repeated restraint?

The results of this experiment are shown in Figure 4.6. No differences were seen between groups in basal ACTH (0 minutes). At 15 and 30 minutes into restraint on day 5, I found that ACTH was reduced in repeatedly restrained animals regardless of intra-BLA drug (15 minutes: Main Effect of Stress: $F(1,38)=11.4$, $p\leq0.001$; 30 minutes: Main Effect of Stress: $(1,37)=9.4$, $p\leq0.005$). There was also a trend at 15 and 30 minutes towards reduced ACTH in animals given intra-BLA MEKI regardless of whether they were given prior repeated restraint (15 minutes: trend towards Main Effect of Drug:

F(1,38)=3.2, p=0.07; 30 minutes: trend towards Main Effect of Drug: F(1, 37)=2.9, p=0.09). At 60 minutes, after recovery from restraint, there was a trend towards HPA activity being reduced in animals given intra-BLA U1026 regardless of whether they were given prior repeated restraint (trend towards Main Effect of Drug F(1,41)=3.2, p=0.07). Next, ACTH values were integrated across timepoints to get a measure of the overall effect of prior restraint and intra-BLA MEK blockade on HPA activity. I found that repeated restraint or repeated intra-BLA U0126 administration reduced integrated ACTH activity to restraint on day 5. (Main Effect of Stress F(1,37)=9.3, p≤0.005, Main Effect of Drug F(1,37)=4.6, p≤0.05). Overall, blocking ERK phosphorylation for 4 days reduced ACTH activation to restraint on day 5, regardless of whether an animal had undergone prior repeated restraint.

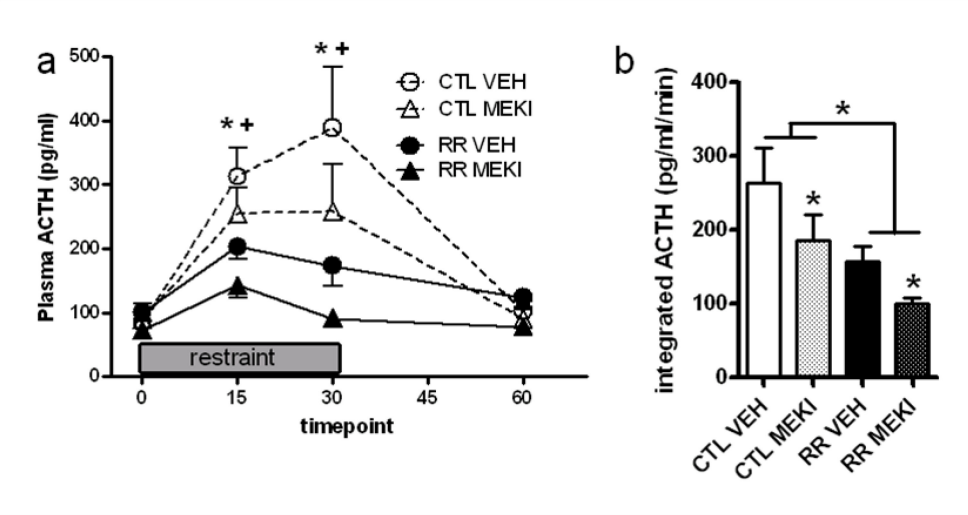

Figure 4.6 ACTH activity in response to restraint after repeated restraint and MEK inhibition in the BLA in Experiment 4.3. (a) depicts the ACTH response to restraint on day 5, and (b) depicts the integrated values for the ACTH response in 4.5(a). In (a), repeatedly restrained (RR) animals had significantly (*) reduced ACTH concentrations in comparison to CTL animals at 15 and 30 minutes into restraint, and animals which received intra-BLA MEK inhibitor (MEKI) tended (+, p ≤ 0.09) to have reduced ACTH concentratins in comparison to VEH animals. In (b), RR significantly reduced integrated ACTH concentration in comparison to CTL, and MEKI significantly reduced ACTH concentrations in comparsion to VEH. Figures depict means ± SEM.

4.4 Discussion

The experiments presented here were designed to test whether changes in intracellular signaling in the BLA in restrained animals contributes to habituation processes. The two intracellular signaling mechanisms explored here, pERK and AcH3, can be regulated

directly by β−AR or by mechanisms regulated by β−AR (Roberson, English, Adams, Selcher, Kondratick, & Sweatt, 1999; Waltereit & Weller, 2003). First, ERK is known to play an important role in learning and memory processes in the BLA (Rossato, Bonini, Coitinho, Vianna, Medina, Cammarota, & Izquierdo, 2004; Schafe, Atkins, Swank, Bauer, Sweatt, & Ledoux, 2000), but its relationship to β−AR activation and its involvement in stress responses was unclear. Second, changes in histone acetylation are known to be regulated by CREB mechanisms that can be regulated by β−AR activation via cAMP and possibly ERK (Oliveira, Wood, McDonough, & Abel, 2007; Roberson, English, Adams, Selcher, Kondratick, & Sweatt, 1999; Waltereit & Weller, 2003). In the hippocampus, histone acetylation changes regulate memory and adaptation to stressors (Chandramohan, Droste, Arthur, & Reul, 2008; Chwang, Arthur, Schumacher, & Sweatt, 2007; Oliveira, Wood, McDonough, & Abel, 2007; Roberson, English, Adams, Selcher, Kondratick, & Sweatt, 1999), but there is very little known about histone modifications in the BLA. I first conducted a pilot experiment measuring changes in pERK and AcH3 in the BLA and hippocampus of restrained animals. Next, I tested whether intra-BLA β−AR blockade after daily restraint prevented these changes. Finally, I tested whether pharmacologically affecting ERK phosphorylation in restrained animals exerted effects on HPA activity to restraint.

Experiment 1	pERK 1	pERK 2	AcH3
US*	–	–	–
CTL	↓	↓	↑
RR	↓	↓	–
Experiment 2	**pERK 1**	**pERK 2**	**AcH3**
CTL VEH*	–	–	–
CTL PROP	–	–	–
RR VEH	↓	↓	↑
RR PROP	–	–	–/↑

Table 4.1 Summary of findings in the BLA from Experiments 4.1 and 4.2. The degree of change in each group of both experiments is in reference to the levels of pERK and AcH3 in the BLA of the groups marked with an asterisk (*). The US group in Experiment 4.1 and the CTL VEH group in Experiment 4.2 can be considered equivalent as neither has received drug treatment or any stress prior to tissue collection. The cell marked with a dash (no difference) and an arrow indicate that there was a trend for this change to have been prevented in the RR PROP group.

There were four major findings in this chapter. First, in Experiments 4.1 and 4.2 restraint induced 1) a decrease in pERK, accompanied by 2) an increase in AcH3, in the BLA. These changes 3) were attenuated in animals given propranolol in the BLA after daily restraint in Experiment 4.2. Finally, 4) pharmacologically blocking pERK in the BLA via inhibition of MEK was sufficient to reduce HPA activity to restraint, regardless of prior stress history, in Experiment 4.3. This pattern of results indicates that decreased ERK phosphorylation in the BLA is an important factor in reducing HPA activity to stressors. Blocking β–AR activation may prevent habituation in animals exposed to repeated stressors animals by in part attenuating this change in ERK activation in the BLA. Increased histone acetylation in the BLA may also be important to habituation to repeated stressors.

In Experiment 4.1, 30 minute restraint reduced ERK phosphorylation in the BLA. There were no significant differences between control rats at the end of acute 30 minute restraint and repeatedly restrained rats at the end of 30 minute restraint in ERK in this experiment. In Experiment 4.2, animals sacrificed under basal conditions that had received prior repeated restraint had reduced ERK phosphorylation in the BLA compared to unrestrained animals. I elected to examine intracellular activity in Experiment 4.2

under basal conditions because Experiment 4.1 indicated that AcH3 was differentially altered by acute versus repeated restraint, and was therefore the most informative dependent measure. However, published literature examining changes in AcH3 in the hippocampus due to stress or conditioning measures basal differences, and for this reason in Experiment 4.2 I sacrificed animals under basal conditions. Because animals were sacrificed basally in Experiment 4.2, the control animals that received vehicle in Experiment 4.2 are most comparable to the unstressed animals in Experiment 4.1, rather than the acutely restrained "control" animals in that experiment. With this comparison in mind, Table 4.1 demonstrates that in both experiments the effect of prior restraint was to reduce tonic pERK in the BLA relative to unrestrained animals, and increase AcH3.

The decrease in pERK as a result of repeated restraint in Experiments 4.1 and 4.2 was unexpected. β−AR activation should result in an activation of cAMP and PKA. Evidence suggests that increased PKA phosphorylation might be expected to lead to increased ERK phosphorylation via two mechanisms. First, in hippocampal culture PKA has been seen to activate a molecule called Rap-1, which leads to increases in pERK (Waltereit & Weller, 2003). Second, phosphorylation of β−AR by PKA has been shown in cardiac cell culture to switch the coupling of the receptor from Gs to Gi (Zou, Komuro, Yamazaki, Kudoh, Uozumi, Kadowaki, & Yazaki, 1999). Gi coupled receptors activate ERK via Ras/Raf proteins (Zou, Komuro, Yamazaki, Kudoh, Uozumi, Kadowaki, & Yazaki, 1999). Significantly, however, Ras/Raf are inhibited by Rap-1 (Waltereit & Weller, 2003), indicating that these two mechanisms of ERK phosphorylation are competitive. Perhaps in the BLA continued stimulation of β−AR by daily restraint results in increased activation of Rap-1. While activation of Rap-1 might lead to increased pERK, Rap-1 could also be inhibiting Ras/Raf, resulting in an overall reduction in pERK in the BLA. Furthermore, because both of these mechanisms linking β−AR to ERK have been observed in non-BLA cells, it is possible that only one mechanism, or neither, occurs in the BLA. Determining whether these intracellular pathways linked to β−AR are occurring or active in the BLA may be helpful in subsequent research.

The comparison between Experiments 4.1 and 4.2 made in Table 4.1 indicates that in both experiments restraint increased AcH3 in the BLA relative to unrestrained animals.

The difference between unstressed (unrestrained) animals and repeatedly restrained animals at the end of restraint in Experiment 4.1 was not significant, as it was in Experiment 4.2 when all animals were sacrificed basally. However, in Experiment 4.1 histone acetylation was significantly greater in acutely restrained controls compared to unrestrained animals. The discrepancy in the effect of prior repeated restraint in between these experiments may be due to the activational effects of restraint in Experiment 4.1. Given that histone acetylation changes can underlie changes in gene expression, it is possible that the gene expression that is being altered by the experience of restraint on day 5 in Experiment 4.1 is different from the gene expression that is being tonically altered by prior repeated restraint in Experiment 4.2. For instance, c-fos gene expression can be mediated by increased histone acetylation (Collins, Hill, Chandramohan, Whitcomb, Droste, & Reul, 2009; O'Donnell, Yang, & Sharrocks, 2008). It is well known that c-fos expression in the telencephalon is elevated by acute restraint and has begun to habituate prior to day 5 (Girotti, Pace, Gaylord, Rubin, Herman, & Spencer, 2006; Weinberg, Girotti, & Spencer, 2007; Weinberg, Bhatt, Girotti, Masini, Day, Campeau, & Spencer, 2008). Thus, the increased histone acetylation in acutely restrained animals in Experiment 4.1 that is no longer significant in repeatedly restrained animals could reflect acetylation related to c-fos expression. In contrast, the tonic changes in histone acetylation induced by prior repeated restraint in Experiment 4.2 could reflect changes in gene expression related to a long-term change in function of the BLA. For instance, the increased BDNF mRNA expression seen in repeatedly restrained animals given intra-BLA vehicle in Chapter 3 could be related to the increased histone acetylation in animals given the same treatment in Experiment 4.2. Future experiments could investigate whether changes in histone acetylation in the BLA of repeatedly restrained animals occurs at BDNF promoter regions. It also remains to be seen whether the changes in histone acetylation seen in repeatedly restrained animals are functionally relevant in the same way that changes in ERK phosphorylation in Experiments 4.1 and 4.2 were shown to be functionally relevant in Experiment 4.3. Subsequent experiments could examine whether pharmacological alterations in BLA histone acetylation could affect HPA responses to stressors such as restraint.

As the decrease in ERK phosphorylation was unexpected, so was the inverse relationship between ERK phosphorylation and histone H3 acetylation in Experiments 4.1 and 4.2. β−AR activation in repeatedly restrained animals should activate PKA, which in turn would phosphorylated CREB and increase overall histone acetylation (Oliveira, Wood, McDonough, & Abel, 2007; Roberson, English, Adams, Selcher, Kondratick, & Sweatt, 1999; Waltereit & Weller, 2003). As discussed above, I hypothesized that repeated restraint would also activate ERK, and there are reports that ERK can also activate CREB (Oliveira, Wood, McDonough, & Abel, 2007; Roberson, English, Adams, Selcher, Kondratick, & Sweatt, 1999; Waltereit & Weller, 2003). Thus, the expected increase in ERK was expected to support increases in histone acetylation mediated by CREB. However, while histone acetylation was found to be increased in repeatedly restrained animals, this increase was associated with a decrease in tonic pERK. There is work in cell culture that has identified a CREB−inhibiting effect of tonic elevations in pERK (Wang, Zhang, Wang, & Carr, 2003). However, work in the hippocampus would suggest that pERK and histone acetylation should be directly related (Roberson, English, Adams, Selcher, Kondratick, & Sweatt, 1999). Contextual fear conditioning is associated with increased pERK, increased acetylated histone H3, and increased phosphorylated histone H3 in CA1 (Chwang, Arthur, Schumacher, & Sweatt, 2007; Levenson, O'Riordan, Brown, Trinh, Molfese, & Sweatt, 2004; Roberson, English, Adams, Selcher, Kondratick, & Sweatt, 1999). Forced swimming increases phosphorylated-acetylated histone H3 in the dentate gyrus, and it was found that blocking ERK phosphorylation in the dentate prevented this increase in phospho-acetyl-H3 (Chandramohan, Droste, Arthur, & Reul, 2008). It is notable that these studies linking ERK and histone acetylation also examined histone phosphorylation. Histone phosphorylation is another form of epigenetic modification that can also increase gene expression. Importantly, histone acetylation is CREB mediated, but histone phosphorylation is mediated by other molecules that are under direct regulation of ERK (Chwang, Arthur, Schumacher, & Sweatt, 2007). The link between ERK and CREB has been established in the hippocampus (Roberson, English, Adams, Selcher, Kondratick, & Sweatt, 1999) but to my knowledge is only assumed in the amygdala (e.g. Schafe et al., 2000). Thus, it is possible that in the BLA of repeatedly restrained animals, pCREB is inversely related to pERK. Indeed, an

investigation on the effect of 15 minutes of forced swimming in limbic brain regions found no change in pERK in the amygdala, but a large increase in pCREB in the same samples (Shen, Tsimberg, Salvadore, & Meller, 2004). If CREB and ERK are dissociated, then it is reasonable to hypothesize that CREB–mediated histone acetylation is dissociated from ERK-mediated histone phosphorylation in the BLA, despite the fact that these factors are coregulated in the hippocampus.

These possibilities, raised by the inverse relationship found here between ERK and histone acetylation indicate that knowing the pattern of PKA phosphorylation, CREB phosphorylation and histone phosphorylation is in the BLA of the animals in Experiments 4.1 and 4.2 would be extremely informative. PKA phosphorylation was not examined in the current experiments, as it is a relatively complicated protein to study with immunohistochemical techniques. Inactivated PKA is comprised of several subunits that dissociate upon phosphorylation, and an activated subunit exerts the effects of PKA (Shobe, 2002). It would be necessary to conduct a number of pilot experiments to determine the subunit or subunits primarily responsible for PKA activation in the BLA, which would have been prohibitive to a timely completion of this dissertation. CREB is by comparison a straightforward protein to measure, and examining pCREB in the BLA of Experiments 4.1 and 4.2 is a high priority that I am currently pursuing. Unfortunately, there is insufficient sample remaining to test histone H3 phosphorylation in the BLA of all animals, although in my time remaining I may attempt to measure it in the subset of animals with BLA nuclear sample remaining.

The results of Experiment 4.3 indicate that the reduction in pERK 1/2 seen in repeatedly restrained rats in Experiment 4.2 is functionally relevant to the regulation of HPA activity in response to restraint. In Experiment 4.3, four days of intra-BLA administration of a MEK inhibitor reduced HPA activity in response to a test restraint. This reduction in HPA activity occurred regardless of whether the animal had received only MEK inhibitor with no prior restraint, or daily post-restraint intra-BLA MEK inhibition. This indicates that in intact animals, habituated HPA activity in response to a homotypic stressor may in part require reduced ERK activity in the BLA. In Figure 4.3c, the combined effects of restraint and drug administration appear to further reduce HPA activity in repeatedly

restrained animals given MEK inhibitor, compared to animals that received MEK inhibitor without daily restraint. Why in Experiment 4.3 is the decrease in HPA activity in response to restraint not equivalent in both groups given intra-BLA MEK inhibitor? The reduction in HPA activity in repeatedly restrained animals given MEK inhibitor below that of animals given MEK inhibitor strongly suggests that HPA activity regulation within the BLA depends on more than pERK. Habituation processes occurring within the BLA probably involves other intracellular mechanisms, possibly including PKA and CREB mediated mechanisms as discussed earlier, as well as unknown mechanisms not related to β-AR. However, the combined results of Experiments 4.1, 4.2, and 4.3 indicate that decreased pERK in the BLA of a repeatedly restrained animal is one mechanism involved in the reduction of HPA activity to a stressor.

In contrast to the restraint-induced differences in ERK phosphorylation and histone acetylation in the BLA, no significant differences were found in the hippocampus in the current experiments. As has already been discussed, here is a great deal of work which suggests that activity in the hippocampus is modified by repeated stressor exposure (Herman, Ostrander, Mueller, & Figueiredo, 2005; McQuade, Tamashiro, Wood, Herman, McEwen, Sakai, Zhang, & Xu, 2006; Sapolsky, 2002; Sapolsky, 2003; Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002), including the results of Chapter 3. Increased ERK phosphorylation and increased histone H3 acetylation in the CA1 region of the hippocampus is necessary for memory consolidation of contextual fear conditioning (Chwang, O'Riordan, Levenson, & Sweatt, 2006; Roberson, English, Adams, Selcher, Kondratick, & Sweatt, 1999; Sweatt, 2001). Similarly, animals that have undergone forced swimming demonstrate increased ERK phosphorylation, and this increased in pERK causes an increase in histone H3 phospho-acetylation (detected by an antibody that cannot distinguish between the phosphorylated form and the acetylated form) in the dentate gyrus of the hippocampus (Chandramohan, Droste, Arthur, & Reul, 2008). The effects of contextual fear conditioning were specific to CA1, and the effects of forced swimming were specific to dentate gyrus, but in Experiments 4.1 and 4.2, the entire dorsal hippocampus was homogenized and analyzed. Thus, changes that might exist in the hippocampus of repeatedly restrained animals in phosphorylated ERK and

acetylated histone H3 may have been obscured in the current experiments by a lack of anatomical specificity in the tissue collection and preparation.

Overall, the results of the current studies indicate that repeated restraint leads to decreased pERK and increased AcH3 in the BLA, and that in the case of pERK these changes are functionally relevant for altering HPA activity in response to stressors. In the overall conclusion, I will discuss the findings of this chapter in conjunction with the previous two chapters, present an updated model of β-AR effects in the BLA, and discuss the overall implications of the experiments in this dissertation.

Chapter 5.

Discussion

5.1 Overview

The overall goal of this dissertation was to examine the role of β-AR signaling in the BLA in regulating habituation to repeated stressors. Habituation occurs in response to repeated experience with a homotypic stressor and results in decreased physiological and behavioral responses to the now-familiar stressor. The BLA is a particular area of interest with regards to habituation. First, the BLA is anatomically linked to other limbic system regions known to regulate stress reactivity (Herman, Ostrander, Mueller, & Figueiredo, 2005; Jaferi & Bhatnagar, 2006; Lang & Davis, 2006). Second, the BLA exhibits increased activity to a novel stressor after prior repeated stressor exposure (Bhatnagar & Dallman, 1998) and exhibits increased dendritic arborization after repeated exposure to a homotypic stressor (Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002; Vyas, Pillai, & Chattarji, 2004). Third, the BLA is well known for its role in regulating learning and memory for aversive or stressful events (Maren, 2005; McGaugh, 2004; Sandi & Pinelo-Nava, 2007). In particular, NE signaling in the BLA via β-AR plays an important role in the consolidation of aversive memories (McGaugh, 2004). Finally, NE signaling in stress-regulatory brain regions other than the BLA regulates HPA activity in acutely and repeatedly restrained animals (Cecchi, Khoshbouei, & Morilak, 2002; Ma & Morilak, 2005; Pardon, Gould, Garcia, Phillips, Cook, Miller, Mason, & Morilak, 2002; Pardon, Ma, & Morilak, 2003). This evidence suggests that NE signaling in the BLA would also regulate HPA responses to stressors. Importantly, the role of NE signaling in the BLA on HPA and behavioral habituation to repeated stressors might occur via β-AR mechanisms, as these mechanisms were already known to influence the degree of behavioral change elicited by an aversive experience.

In light of this evidence, I hypothesized that the BLA would regulate HPA and behavioral responses to the repeated stressor of restraint via β-AR signaling mechanisms similar to those described by McGaugh and colleagues. To approach this question, I examined the effects of β−AR manipulations after daily restraint on a number of measures of habituation. In Chapter 2, I determined whether post-restraint, intra-BLA β-AR manipulations affected HPA and behavioral habituation to repeated restraint. In Chapter 3, I determined whether these β-AR manipulations prevented changes in gene expression in stress-response-regulatory brain regions. In Chapter 4, I examined whether repeated restraint and β-AR blockade induced changes in intracellular activation of ERK and histones, and examined whether the change in ERK activation was functionally relevant.

In the first section of this discussion chapter, I will go into the major findings of each chapter, and relate the findings of each chapter to each other. In the second section, I will discuss the current findings in light of the broader literature, as part of a discussion of the theoretical implications of the current work on 1) the BLA, NE, and intracellular signaling, 2) the relationship between HPA habituation and models of learning and memory, and 3) propranolol in the clinical treatment of post-traumatic stress disorder.

5.2 Major findings

In Chapter 2, I tested the main hypothesis of this dissertation. β-AR activation in the BLA after restraint was hypothesized to be necessary for the acquisition of habituation to restraint on days 1-4. Thus, β-AR blockade in the BLA after daily restraint on days 1-4 would prevent the expression of habituation in response to restraint on day 5. There were four major findings in this chapter. 1) Immediate post-restraint administration of the β-AR antagonist propranolol in the BLA prevented habituation of HPA activity to the 5th restraint, but 2) propranolol administered 4 hours after daily restraint did not prevent habituation to the 5th restraint. 3) Post-restraint intra-BLA propranolol prevented the habituation of struggling behavior to repeated restraint. 4) The β−AR agonist clenbuterol administered in the BLA after daily restraint enhanced the habituation of HPA activity in response to restraint on days 3 and 5. These results establish that habituation to repeated restraint is modified by intra-BLA β-AR manipulations post-restraint. The direction of

these effects parallel the effects of β-AR manipulations on the consolidation of inhibitory avoidance conditioning. These results are novel not only because they 1) establish a role for NE signaling in the BLA in regulating responses to repeated stressors, but because 2) these results suggest an explicit link between stress response habituation and learning and memory. Experiments in Chapters 3 and 4 further addressed the first claim by examining changes in gene expression and intracellular signaling in stress-response-regulatory brain regions as a result of repeated restraint and intra-BLA β-AR blockade. Arguments for and against the second claim will be discussed later in this chapter.

In Chapter 3, I examined changes in stress-related gene expression induced by repeated restraint at the end of 30 minute restraint on day 5, and asked if these changes could be prevented by intra-BLA β-AR blockade with propranolol after daily restraint. There were three major findings in this chapter. 1) AVP mRNA in the PVN was increased in repeatedly restrained animals, but intra-BLA propranolol after daily restraint prevented this increase in AVP. 2) Repeated restraint led to increased BDNF mRNA in the basal portion of the BLA, but daily β-AR blockade prevented this increase. 3) In CA3 and dentate gyrus, BDNF mRNA was increased in animals that received repeated restraint relative to control animals given vehicle in the BLA. In addition, dentate gyrus BDNF mRNA was increased by β-AR antagonist alone, without repeated restraint.

The combination of results from Chapters 2 and 3 suggest that under normal conditions, restraint-induced β-AR activation in the BLA alters signaling from the BLA that ultimately drives the PVN to stimulate HPA activity. The blockade of habituation in repeatedly restrained animals given the β−AR antagonist paralleled the blockade of increased AVP mRNA in the PVN in animals given this treatment. This indicates that plasticity in stress-response-regulatory circuits between the BLA and the PVN regulates experience-dependent changes in HPA activity, behavior, and plasticity in the PVN. The BLA does not directly impinge on the PVN, but most directly connects via the BNST (Lang & Davis, 2006). Parsimoniously, it may be that β-AR activation in stressed animals alters synaptic connectivity between the BLA and nuclei within the BNST, a possibility that could be tested in future experiments. As the habituation of struggling behavior was also prevented by intra-BLA propranolol, there are likely repeated stressor-

induced alterations in plasticity between the BLA and other brain regions that mediate struggling, though these are unknown. Examination of neuronal activity in repeatedly restrained animals given intra-BLA propranolol, for example by looking at c-fos mRNA in the animals in Chapter 3, might indicate structures that are especially important to regulating responses to repeated stressors.

Chapters 2 and 3 in conjunction also suggest that β-AR activation within the BLA of repeatedly restrained rats supports the increase of BDNF mRNA expression in this region. The change in BDNF mRNA in the BLA in Chapter 3 parallels the change in HPA activity in Chapter 2. This suggests that the increase in BDNF in the BLA may be important or necessary for habituation to repeated restraint. As suggested in Chapter 3, future experiments could infuse BDNF into the BLA after daily restraint to determine if this enhances habituation. The fact that β−AR blockade prevented stressor-induced increases in BDNF mRNA in the BLA suggests that β-AR blockade may prevent intracellular changes that are important to altering gene expression. These changes in intracellular signaling may be involved in the process of habituation to repeated stressors. This idea was explored in Chapter 4.

In Chapter 4, I examined intracellular changes within the BLA and hippocampus to determine the effect of restraint and intra-BLA β-AR blockade on signaling in these regions. I tested pERK and AcH3 protein in each region. The former is the activated form of the intracellular second messenger ERK, which could be activated by β-AR and which is necessary for Pavlovian and operant aversive learning (Rossato, Bonini, Coitinho, Vianna, Medina, Cammarota, & Izquierdo, 2004; Schafe, Atkins, Swank, Bauer, Sweatt, & Ledoux, 2000). The latter is an epigenetic modification of the histone H3 that is important to learning in the hippocampus (Chandramohan, Droste, Arthur, & Reul, 2008; Levenson, O'Riordan, Brown, Trinh, Molfese, & Sweatt, 2004) that could be induced by β-AR activation of CREB. There were four major findings from this chapter, all occurring within the BLA. 1) Restraint decreased pERK in the BLA, but 2) intra-BLA β-AR blockade after daily restraint prevented this reduction in pERK. Along with this, 3) restraint increased AcH3 in the BLA, but intra-BLA β-AR blockade attenuated this increase. 4) MEK inhibitor, which prevents MEK phosphorylation of ERK, infused in

the BLA was sufficient to reduce HPA activity to restraint on day 5, regardless of whether MEK inhibitor was delivered after daily restraint on days 1-4, or alone without restraint on days 1-4.

The increase in BDNF mRNA in repeatedly restrained animals demonstrated in Chapter 3 is interesting in light of the results from Chapter 4. Increased histone acetylation increases the availability of DNA for transcription. Increased BDNF mRNA in the BLA of repeatedly restrained animals may be a result of increased transcription of BDNF exons via increased histone acetylation. Repeated stressors have previously been shown to lead to changes in BDNF exon transcription via changes in histone acetylation in the hippocampus (Tsankova, Berton, Renthal, Kumar, Neve, & Nestler, 2006). Future studies could examine the relationship between histone acetylation and HPA activity by inhibiting histone deacetylation in the BLA of repeatedly restrained animals. If the pharmacological increase in AcH3 induced by deacetylase inhibitors reduced HPA activity, as inhibition of pERK did in Experiment 4.3, this would suggest that increased histone acetylation in the BLA is required for HPA habituation. A further experiment could be done to determine if histone acetylation around BDNF coding regions is altered in the BLA of repeatedly restrained animals.

In conjunction with the results of Chapter 2, Chapter 4 indicates that habituation to repeated restraint is associated with a tonic decrease in pERK that is regulated by β−AR activation. This suggests that the repeatedly restrained animals that exhibited decreased pERK would have exhibited habituated HPA activity had they been challenged with restraint. Furthermore, inhibiting MEK, which leads to decreased pERK in the BLA, decreased HPA activity in response to restraint, regardless of whether an animal had received prior repeated restraint. This suggests that β-AR mediated reductions in pERK are part of the mechanism by which β-AR activation leads to habituation of HPA activity to repeated restraint. The negative regulation of pERK by β−AR activity has not been previously demonstrated, and was unexpected. As the results of Chapter 2 suggest that at memory consolidation mechanisms may be involved in HPA habituation to repeated stressors, and memory consolidation of aversive conditioning is associated with increased pERK, one might expect pERK to be increased in the BLA of a habituated animal. While

the reasons for this unexpected result are unclear, it has implications both for the biological mechanisms involved in β-AR actions in the BLA, and for the similarities and differences between habituation to repeated stressors and paradigms of aversive learning and memory. These implications are addressed in the next section.

5.3 Implications of current findings in light of broader literature

The results of this dissertation have implications at two major levels of analysis. At a micro level, I believe the operational model of β−AR actions in the BLA presented in Chapter 1 should be modified to incorporate the current findings. At a macro level, the results of this dissertation suggest that habituation to repeated stressors has certain similarities and differences with models of learning and memory.

Updated model of NE actions in BLA

I designed the experiments in this dissertation based on the model of β-AR actions in the BLA as proposed by McGaugh and Roozendaal, who have lead the field in characterizing the effects of pharmacological manipulations on the BLA on consolidation of memory for inhibitory avoidance learning. McGaugh and Roozendaal's model is shown in Figure 5.1 (from Roozendaal, Schelling, and McGaugh, 2008). In this model, the crucial function of β−AR activation is to stimulate cAMP and PKA. PKA has unknown actions downstream that alter memory consolidation since blocking PKA is detrimental to memory consolidation and prevents the actions of β-AR activation (Roozendaal, Quirarte, & McGaugh, 2002; Roozendaal, Schelling, & McGaugh, 2008; Tronson, Wiseman, Olausson, & Taylor, 2006). α1-AR, CRF, and glucocorticoids acting via GR enhance β−AR signaling to cAMP (Ferry, Roozendaal, & McGaugh, 1999; Roozendaal, Quirarte, & McGaugh, 2002; Roozendaal, Schelling, & McGaugh, 2008). McGaugh, Roozendaal, and colleagues (Roozendaal, Brunson, Holloway, McGaugh, & Baram, 2002; Roozendaal, Schelling, & McGaugh, 2008) mention that in addition to enhancing β−AR activation of cAMP, glucocorticoids do have some actions outside of the cell which might further enhance NE effects on the BLA, including potentiating NE release from terminals and preventing NE uptake by glial cells (for review, see Roozendaal, Schelling,

and McGaugh, 2008). Finally, α2-AR, identified as presynaptic autoreceptors, act to oppose the effects of β-AR by inhibiting NE release from the terminal.

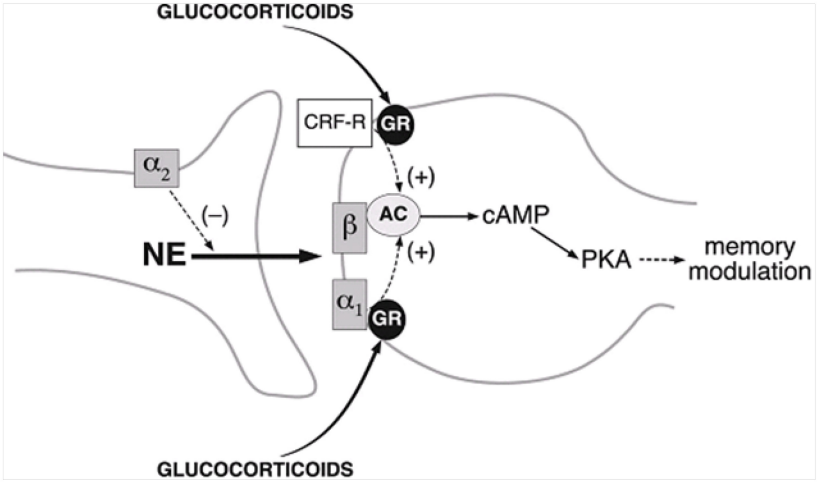

Figure 5.1 McGaugh and Roozendaal model of β–AR actions in the BLA. From Roozendaal, Schelling, and McGaugh, 2008. In this model, cAMP activation by β–AR is the crucial step mediating the effects of β–AR stimulation. It does not account for the overall inhibition of neurons by NE as demonstrated by Buffalari and Grace (2007, 2009), or for the reduction in pERK in the BLA associated with β-AR activation in repeatedly restrained rats as seen in Chapter 4. See text for more details.

Applying McGaugh and Roozendaal's model to the results presented in this dissertation, it appears that some results are consistent with the model, but other results are not addressed by the model. Consistent with McGaugh and Roozendaal's model, β-AR blockade after "training" (i.e., daily restraint) prevented the acquisition of changes in HPA activity and behavior to restraint. This model also provides implied support for the increase in histone acetylation in Chapter 4, as activated PKA can phosphorylate CREB, and phosphorylated CREB increases histone acetylation. Lastly, this model would suggest that glucocorticoid release induced by daily stressors can potentiate the actions of NE on β-AR, enhancing the habituation of HPA and behavioral responses to stressful, glucocorticoid-releasing events.

I propose an update to McGaugh and Roozendaal's model, shown in Figure 5.2. McGaugh and Roozendaal's model does not address the decrease in pERK due to restraint seen in Chapter 4, and the mechanisms proposed in Figure 5.2 are meant to add to their model to incorporate the current findings in a parsimonious fashion. It could be argued that changes in pERK induced by β-AR are not consequential unless pERK

interferes with PKA signaling. In fact, there is evidence to support the idea that pERK interferes with PKA signaling to CREB, because tonic increases in pERK have been found to inhibit CREB (Wang, Zhang, Wang, & Carr, 2003). Because of this evidence, I argue that pERK is consequential to the effects of β-AR in the BLA, suggesting functional significance to the relationship between β-AR and pERK established in Chapter 4. The current data would suggest that PKA and ERK in the BLA may be competitive. A mechanism for this competition is diagrammed in Figure 5.2. As suggested in Chapter 4, PKA-mediated increases in Rap-1 may inhibit Ras/Raf-mediated pERK (Waltereit & Weller, 2003; Zou, Komuro, Yamazaki, Kudoh, Uozumi, Kadowaki, & Yazaki, 1999). At the same time, ERK may inhibit downstream PKA effects by inhibiting CREB (Wang et al., 2003). These possibilities could be tested via Western blot of pCREB and Rap-1 in the BLA, and examining the effects of intra-BLA antagonism of ERK and PKA on some of these intracellular messengers.

It should be noted that the model in Figure 5.2 assumes that the pERK differences observed in the current experiments and the cAMP/PKA effects found by McGaugh and others occur in the same cell. This assumption is rooted in a lack of anatomical specificity in intra-BLA drug administration used in this dissertation and the literature, and in the Western blot technique used in Chapter 4. The BLA contains a variety of neuronal subtypes, including both inhibitory interneurons and excitatory projection neurons (Buffalari & Grace, 2007; Buffalari & Grace, 2009; Muller, Mascagni, & McDonald, 2006; Muller, Mascagni, & McDonald, 2009). It is possible that the changes occurring in pERK in the BLA occur in a different population of neurons from those altered by PKA manipulations. This would indicate that a complete model of NE actions in the BLA would need to incorporate these different neuronal subtypes, and detail their interactions. NE induces heterogeneous responses in BLA neurons, inhibiting most but exciting some, and in fact, repeated stressor exposure can increase the ratio of excitatory to inhibitory responses elicited by NE (Buffalari & Grace, 2007; Buffalari & Grace, 2009). This suggests that activation patterns of inhibitory and excitatory neurons in the BLA may be differentially altered as a result of repeated stressor exposure. Subsequent experiments could investigate patterns of activation in BLA interneurons versus BLA projection neurons by repeated restraint and by NE, and could investigate the neuronal subtype

specificity of pERK changes versus cAMP/PKA changes induced by repeated restraint and β-AR activation. It may also be the case that habituation to repeated restraint involves similar β-AR mechanisms as those proposed by McGaugh and Roozendaal, but the role of pERK differs between operant avoidance and stress response habituation. This possibility is discussed in the next section. However, the model presented in Figure 5.2 represents a parsimonious viewpoint of the relationship between β-AR manipulations and pERK by presenting these changes within a single cell.

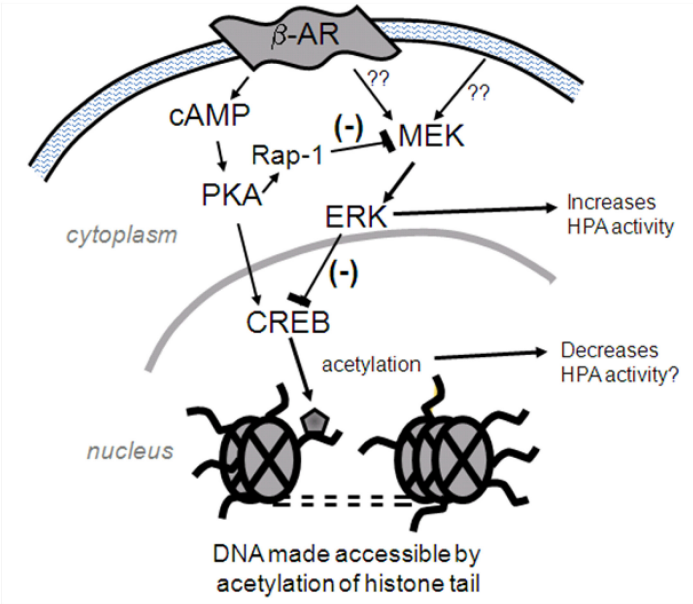

Figure 5.2 Revised model of β-AR actions in the BLA. It includes the hypothesized competitive relationship between PKA and ERK effects in the cell suggested by the results of Chapter 4, and as described in this chapter. CRF, GR, and α-AR effects from Figure 5.1 were not included to simplify this figure, though they are still presumed to occur. pERK is known to increase HPA activity as inhibiting pERK decreases HPA activity (Chapter 4). Histone acetylation is presumed to decrease HPA activity as it is increased in the BLA of repeatedly stressed animals that did not receive β-AR blockade (Chapter 4). See text for further detail.

Finally, while the data presented here supports a modification of the model used to support the role of β-AR in memory consolidation, it should be noted that some relatively recent evidence has been interpreted as indicating that NE is not necessary for the consolidation of emotional memory (Murchison, Zhang, Zhang, Ouyang, Lee, & Thomas, 2004). The evidence that NE is not necessary for consolidation comes from a series of experiments in mice that are unable to synthesize NE postnatally (Murchison, Zhang, Zhang, Ouyang, Lee, & Thomas, 2004; Thomas & Palmiter, 1998). These mice do

display deficiencies in contextual fear conditioning. However, these deficiencies are rescued by systemic NE precursor (L-DOPS) administration prior to retrieval, not prior to training, a finding interpreted as demonstrating that NE is not necessary for consolidation (Murchison, Zhang, Zhang, Ouyang, Lee, & Thomas, 2004). While the importance of NE signaling in memory retrieval is not in dispute, there are some methodological issues in the study by Murchison and colleagues that may explain the lack of effect of NE replacement on consolidation.

First, the use of mutant mice in the majority of the experiments in Murchison and colleagues limits the interpretability of the data. While NE was found necessary for the retrieval, but not the consolidation, of memories for contextual fear conditioning in mutant mice, when testing whether these effects held in outbred rats, only the role of NE in retrieval was tested, but not the role of NE in consolidation (Murchison, Zhang, Zhang, Ouyang, Lee, & Thomas, 2004). It is possible that the effects seen in this study on consolidation may be limited to this mutant mice population, which may have subtle neurodevelopmental differences or deficits as compared to normal animals. Second, the effect of NE precursor replacement in the mutant mice was tested by a single intraperitoneal injection of precursor or vehicle prior to training (manipulating consolidation) or testing (manipulating retrieval). Intraperitoneal injections are stressful (Spencer & McEwen, 1990) and would lead to HPA activation and glucocorticoid release. Intermediate doses of glucocorticoids can enhance both consolidation and recall as compared to very low or high doses (Roozendaal, Quirarte, & McGaugh, 2002; Roozendaal, Hahn, Nathan, de Quervain, & McGaugh, 2004; Roozendaal, Okuda, de Quervain, & McGaugh, 2006). It is not known whether these mutant mice have altered HPA function resulting from the genetic manipulation, which could lead to alterations in glucocorticoid release related to injection and drug administration that could exert important effects on consolidation and retrieval. Third, the dose-response function of glucocorticoids on memory consolidation recalls the dose-response function of NE in memory consolidation, as has been demonstrated by McGaugh and colleagues (Roozendaal, Quirarte, & McGaugh, 2002; Roozendaal, Schelling, & McGaugh, 2008) and in Chapter 2 of this dissertation. It is quite possible that the levels of NE activity necessary for optimal memory consolidation are different than those that are optimal for

memory retrieval. As the NE precursor was only given in one dose, this dose may have been ideal for enhancing retrieval, but too high or low to enhance consolidation. Addressing these methodological caveats with additional experimentation would go far to reconcile the discrepancies between the findings of Murchison and colleagues (2004) and the many previous findings that NE is important for consolidation (McGaugh, 2004).

Relationship between habituation to repeated stressors and models of aversive learning

In this discussion of the implications of this work, the first section addressed the McGaugh and Roozendaal model of NE action in the BLA and modified it to account for the results obtained in this dissertation. In that section, as in the entire dissertation, hypotheses of how habituation to repeated stressors would be affected by intra-BLA pharmacological manipulations were based on how these intra-BLA pharmacological manipulations in the BLA altered memory consolidation for aversive learning, especially operant inhibitory avoidance conditioning. This approach was fruitful with regards to the role of β−AR in the BLA. β-AR antagonist and agonist effects on habituation to repeated stressors paralleled the effects of these compounds on memory consolidation for inhibitory avoidance learning (McGaugh, 2004). However, the effects of MEK inhibition on habituation to repeated restraint in Chapter 4 were the opposite of what has been found for inhibitory avoidance learning (Rossato, Bonini, Coitinho, Vianna, Medina, Cammarota, & Izquierdo, 2004). This is a discrepancy if one assumes that habituation to repeated stressors involves similar memory consolidation mechanisms as operant aversive paradigms, an assumption at the root of this dissertation. As part of attempting to address this assumption and the resulting discrepancy, it is necessary to discuss the similarities and differences between habituation to repeated stressors and these models of aversive learning and memory.

The process of habituation to repeated stressors is, in part, regulated by glucocorticoid negative feedback mechanisms, acting on MR and GR in the brain and pituitary (Dallman et al., 1987). Systemic blockade of MR before a test restraint elevates HPA activity and prevents the expression of habituation in animals that received prior repeated restraint (Cole et al., 2000). Our lab has shown that specific daily blockade of MR and GR in the paraventricular thalamus before daily restraint prevents the development of habituation to

repeated restraint (Jaferi & Bhatnagar, 2006). If glucocorticoids or GR agonists in the BLA enhance the habituation of HPA activity to repeated restraint, this could be considered both a negative feedback effect, and support the McGaugh and Roozendaal model in habituation to repeated restraint in via GR enhancement of β-AR activation. Thus, if HPA habituation is subject to memory consolidation mechanisms in the BLA, glucocorticoid negative feedback may support these mechanisms. However, glucocorticoid negative feedback cannot fully account for the phenomenon of HPA habituation. Adrenalectomized animals, which lack stress-induced negative feedback produced by glucocorticoids, are still able to habituate (Jaferi & Bhatnagar, 2006). Thus, there are mechanisms in the brain that reduce HPA activity to a familiar stressor independent of glucocorticoid signaling.

The neural mechanisms that reduce HPA activity to a familiar stressor are probably involved in evaluating whether the current stressor is familiar or not, as this evaluation appears to be crucial to whether or not a habituated response is produced. For instance, rats habituate to restraint even while simultaneously undergoing other, variable stressors, to which they do not habituate (Simpkiss & Devine, 2003) indicating that the HPA habituation is specific to the familiar homotypic stressor. Furthermore, habituation to restraint is context specific, because a change in the odor and location of restraint on day 8 compared to the previous 7 days of restraint leads to a dishabituation of HPA activity (Grissom, Iyer, Vining, & Bhatnagar, 2007). Ultimately one or more brain regions are involved in evaluating whether the current stressor is similar to a previous nonharmful experience, and reduce HPA activity, or dissimilar from previous experiences and therefore potentially threatening, thereby activating HPA activity. This hypothesized evaluation process makes use of previous learning about stressor experiences, learning which could be nonassociative or associative in nature. That is, habituation to repeated stressors could be an example of nonassociative learning such as response habituation (Groves & Thompson, 1970; Pitman, Ottenweller, & Natelson, 1990; Thompson & Spencer, 1966), or it could be regulated by associative learning (Fanselow & Poulos, 2005; Maren, 2003; Maren, 2005), or both, discussed below.

Earliest use of the word "habituation" to refer to decrements in HPA activity to a familiar stressor (Hennessy & Levine, 1977; Pfister, 1979) corresponded with the acknowledgement that this reduction could be "response habituation" as defined by Richard Thompson and colleagues (Groves & Thompson, 1970; Thompson & Spencer, 1966). "Habituation" in the sense indicated by Thompson and colleagues refers to any decrease in responsiveness to a repeated stimulus, a form of nonassociative learning. Thompson and Spencer (1966) forwarded a set of criteria to provide a "functional definition" of response habituation, which can be used to evaluate decrements in response to determine if they are also examples of response habituation. I recently reviewed the literature on HPA habituation as a result of repeated stress in light of these criteria to determine whether the use of "habituation" to describe this phenomenon was appropriate – in other words, to determine if changes in HPA activity due to repeated stress reflect nonassociative learning (Grissom and Bhatnagar, 2008). To summarize that review here, I found evidence in the literature to support some, but not all, of Thompson's criteria. Supported criteria included 1) the basic phenomenon of reduced responses (habituation itself) of the HPA axis to repeated homotypic stressors. 2) HPA habituation is subject to dishabituation by a novel stressor. 3) HPA habituation is greater to milder stressors than more severe stressors. 4) HPA habituation is greater to repeated stressors with a shorter interstress interval than a longer interstress interval. However, reductions in HPA activity to a homotypic stressor do not meet a number of Thompson's criteria, which call into question response habituation as the only explanation of HPA habituation other than glucocorticoid negative feedback. Habituated HPA activity does not spontaneously recover (Armario, Valles, Dal-Zotto, Marquez, & Belda, 2004; Bhatnagar, Huber, Nowak, & Trotter, 2002; Marti, Garcia, Valles, Harbuz, & Armario, 2001; Vogel & Jensh, 1988) which must be observed to evaluate three of Thompson's nine criteria. In fact, HPA reductions to a repeated stressor appear if anything to become greater over time (Marti, Garcia, Valles, Harbuz, & Armario, 2001; Vogel & Jensh, 1988). HPA habituation also appears to be highly stimulus specific, and does not generalize to other stressors (Grissom, Iyer, Vining, & Bhatnagar, 2007; Simpkiss & Devine, 2003), violating Thompson's criterion of stimulus generalization. These results suggest that there are probably nonassociative learning mechanisms involved in the habituation of

HPA activity to repeated stressors. The lack of spontaneous recovery and the lack of stimulus generalization of HPA habituation indicate that more specific, long-term, and/or associative mechanisms may also be involved.

Changes in HPA activity elicited by a familiar stressor, in addition to hinging on mechanisms of negative feedback and response habituation, might be related to associative learning mechanisms. Several pieces of evidence support this idea. Habituation of HPA activity can be disrupted by a change in contextual cues, suggesting contextual learning (Grissom et al., 2007). HPA activity in stressful environments is reduced by exposure to a conditioned inhibitor or signal of stressor termination (Arnhold, Wotus, & Engeland, 2007; Campeau, Falls, Cullinan, Helmreich, Davis, & Watson, 1997). HPA activity can be increased by a conditioned stimulus (Levine, Smotherman, & Hennessy, 1977). Furthermore, HPA activity has been shown to be reduced to only the second exposure to a stressor despite a long interval of time between the first and second exposures (Armario et al., 2004; Vogel & Jensh, 1988). This suggests that some sort of memory trace produced by prior stressor exposure may influence subsequent HPA activity (Armario, 2006). Finally, habituation of HPA activity involves activation of distributed limbic circuitry (Herman & Cullinan, 1997; Herman, Figueiredo, Mueller, Ulrich-Lai, Ostrander, Choi, & Cullinan, 2003; Herman, Ostrander, Mueller, & Figueiredo, 2005) that overlaps with circuitry important for associative learning (Davis, 2006; Hunt, Fanselow, Richardson, Mauk, Freeman, & Stanton, 2007; Lang & Davis, 2006; Walker & Davis, 2008).

The evidence cited above supports the idea that HPA activity might be subject to associative learning, but does not ascribe a particular model of associative learning to HPA habituation. The experiments in this dissertation were modeled after experiments demonstrating BLA effects on operant aversive paradigms. The reason for this approach was not driven by theoretical concerns, but from the observation that BLA lesions did not exert profound effects on HPA activity (Bhatnagar, Vining, & Denski, 2004; Carter, Pinnock, & Herbert, 2004). Because BLA lesions profoundly diminish the ability of the animal to form Pavlovian associations (Zimmerman, Rabinak, McLachlan, & Maren, 2007) but has a more limited effect on operant associations (McGaugh, 2004), the role of

the BLA HPA habituation was presumed to be more related to its role in operant memory consolidation. The most significant of the distinctions between BLA effects on Pavlovian versus operant aversive learning concerns the effects of post-training BLA manipulations. Post-training BLA manipulations affects the consolidation of operant aversive learning, and the *reconsolidation* of Pavlovian conditioning, but has no effect on the original consolidation of Pavlovian aversive learning. Post-stressor ("training" in this dissertation) intra-BLA manipulation of β-AR affected subsequent stress responses. The similarity between the effect of post-"training" BLA β-AR manipulations on habituation to repeated restraint and operant avoidance supports the assumption that the role of the BLA in HPA habituation may be more related to operant memory mechanisms than Pavlovian memory mechanisms.

However, the results of this dissertation indicate that the habituation of HPA activity to a homotypic stressor, while regulated by intra-BLA signaling, does not parallel all literature on the role of the BLA in operant aversive learning. The results of Chapter 4 indicate that decreased pERK in the BLA is associated with decreased HPA activity. This is in direct contrast with the literature suggests that inhibition of pERK in the BLA prevents the consolidation of operant avoidance learning (Izquierdo et al, 2004). There are mechanistic reasons why pERK may be differentially regulated in repeatedly stressed versus aversively conditioned animals, and it is possible that this difference may reflect different psychological processes.

The difference in the direction of change in pERK elicited by repeated stressors versus fear conditioning paradigms could me mediated via several mechanisms. First, it should be emphasized that the increase in pERK in the BLA of animals which undergo fear conditioning is temporary (Schafe, Atkins, Swank, Bauer, Sweatt, & Ledoux, 2000), in contrast with the tonic decrease shown in Chapter 4. The temporal dynamics of pERK in the BLA of animals that have been stressed versus exposed to Pavlovian conditioning may differ. Second, decreased tonic pERK could reflect nonassociative habituation processes, whereas the increases in pERK in the BLA may be required for associative learning. If associative learning processes are involved in habituation to repeated stressors, it may be mediated by increases in other intracellular messengers such as PKA.

Third, the decrease in pERK may be occurring in a different population of neurons, for instance interneurons, than the projection neurons that express increased pERK in models of aversive learning. This possibility provides yet another incentive to establish the neuronal subtypes expressing changes in pERK in repeatedly stressed animals.

The psychological significance of pERK decreases in repeatedly stressed animals versus increases in aversively trained animals may be related to a differential role for BLA pERK in fear versus anxiety. There is recent literature suggesting that levels of pERK in the BLA are directly related to levels of anxiety (Botreau & Gisquet-Verrier, 2006; Wu, Hsu, Tu, Wang, Huang, Pawlak, & Ho, 2008). Infusions of d-cycloserine into the amygdala, a partial NMDA receptor agonist, increased ERK phosphorylation in the amygdala and decreased time spent on the open arm of the elevated plus maze, indicating increased anxiety (Wu, Hsu, Tu, Wang, Huang, Pawlak, & Ho, 2008). In a different experiment, animals trained over 15 trials to express avoidance in response to a light cue had decreased pERK in the amygdala when exposed to the cue compared to uncued animals (Botreau & Gisquet-Verrier, 2006). Animals that are well-trained to perform avoidance in response to a cue experience reduced anxiety in response to the cue (Mineka & Gino, 1980). Thus, the animals in Botreau and Gisquet-Verrier's experiment that exhibited reduced BLA pERK in response to the cue may have been trained on avoidance to the point that their anxiety to the cue was reduced as well. It is possible that reductions in struggling behavior elicited by restraint (Chapter 2, Appendix A) is indicative of reductions in anxiety. Therefore, decreased pERK in repeatedly restrained animals in Chapter 4 may mediate decreased anxiety as measured by decreased struggling behavior in these animals in Chapter 2.

Although the decrease in pERK may reflect changes in innate anxiety, the overall effect of β-AR manipulations in the BLA suggest that memory consolidation processes may play a role in habituation to repeated stressors. While I do not believe that it is fundamentally problematic to invoke memory consolidation literature to describe some processes involved in habituation to repeated stressors, it must be emphasized that HPA habituation is also regulated by glucocorticoid negative feedback and nonassociative learning. Perhaps the most difficult aspect of exploring reductions in HPA activity as a

learned phenomenon is the apparent complexity of the contextual cues that signal whether a stressor is familiar. In the current experiments, I have not attempted to tease apart which contextual or cue stimuli are more or less important to HPA adaptation to repeated stressors, nor have I attempted to determine the perceptual or computational role of any brain structure in terms of connecting the stimulus to the "conditioned" response. In repeated restraint, I made use of a stressor that is widely used in the stress literature (Jaferi & Bhatnagar, 2007; Marin, Cruz, & Planeta, 2007; Reznikov, Reagan, & Fadel, 2008; Weinberg, Girotti, & Spencer, 2007; Weinberg, Bhatt, Girotti, Masini, Day, Campeau, & Spencer, 2008), with which our laboratory had considerable experience (Bhatnagar, Huber, Nowak, & Trotter, 2002; Bhatnagar & Vining, 2003; Bhatnagar, Vining, & Denski, 2004; Jaferi & Bhatnagar, 2006; Jaferi & Bhatnagar, 2007; Vining, Iyer, & Bhatnagar, 2007), and for which I was able to identify a behavioral endpoint to bolster the HPA results (Grissom, Kerr, & Bhatnagar, 2008; Appendix A). However, any subsequent studies that wish to more explicitly connect HPA activity to conditioned responses may want to make use of paradigms with fewer contextual variables, or examine HPA activity in an established learning paradigm, to begin to identify the roles of these variables in generating the response.

In sum, current evidence indicates that "habituation" of HPA activity is more complicated than its name implies. The adaptive reduction of responses to repeated stressors appears to involve complex interactions between negative feedback mechanisms induced by repeated stressor-induced release of glucocorticoids, response habituation mechanisms produced by repeated exposure to the stressor, and memory regarding previous stressor exposures. A full understanding of habituation to repeated stressors will likely depend on understanding the relationships between all of these mechanisms.

Clinical implications

It is important to identify the neural mechanisms involved in changes in HPA activity to repeated stressors for a number of reasons. Stressor-induced HPA activation is a metabolically costly response system, with potentially deleterious effects if overactive. Therefore, it is adaptive for an organism to reduce HPA activity to a stressor that is not inherently harmful (Dallman et al., 1987; McEwen, 2004; Nesse et al., 2007). HPA

responses are disrupted in persons suffering from psychopathology including major depression and post traumatic stress disorder (PTSD) (Golier et al., 2007; Simeon et al., 2007; Thomson & Craighead, 2007; Yehuda et al., 1996) Better understanding of the mechanisms that regulate HPA activity could likely aid in understanding the etiology and treatment of these and other disorders.

In addition to the literature discussing the relationship between adaptation to stressors and psychological disorders, there is a developing clinical literature of the effect of propranolol administration in patients suffering from PTSD. The findings in this dissertation suggest an interesting intersection between these literatures. To begin I will briefly review the propranolol – PTSD literature. The interest in propranolol as a treatment for PTSD is based on the idea that reducing the strength or salience of the traumatic memory by disrupting memory consolidation or reconsolidation will alleviate PTSD symptoms, such as intrusive memory of the traumatic event (Yehuda & LeDoux, 2007). PTSD is defined as the continuation of these symptoms beyond the first month after the trauma, but experiencing these symptoms in the first days and weeks post-trauma is normal. There is limited evidence that propranolol can reduce symptoms of PTSD (Pitman, Sanders, Zusman, Healy, Cheema, Lasko, Cahill, & Orr, 2002; Vaiva, Ducrocq, Jezequel, Averland, Lestavel, Brunet, & Marmar, 2003). Patients who opted to take propranolol in the emergency room following their traumatic event appeared to have reduced PTSD symptoms two months following the event as compared to patients who refused propranolol (Vaiva, Ducrocq, Jezequel, Averland, Lestavel, Brunet, & Marmar, 2003). A similar study of patients recruited in the emergency room which were assigned propranolol or placebo in a double-blind trial found that receiving propranolol for a total of 10 days, with the first dose given 6 hours after the trauma, reduced autonomic responses elicited by script-driven imagery of the traumatic event when tested 3 months later (Pitman, Sanders, Zusman, Healy, Cheema, Lasko, Cahill, & Orr, 2002). These studies are suggestive but ultimately limited by the low incidence of PTSD in their control groups. This is reflective of the overall 6.8% rate of PTSD in the population at large (Yehuda & LeDoux, 2007), despite the fact that lifetime prevalence of experiencing trauma as defined by DSM-IV is much higher (Breslau & Kessler, 2001). As most people experiencing trauma will not develop PTSD, and as administration of propranolol within

the consolidation window in a clinical setting presents logistical concerns, it may be more effective to examine the role of propranolol on preventing reconsolidation after memory re-exposure in an established PTSD population. In such an experiment, PTSD patients given a single dose of propranolol before recall of the traumatic event via script-driven imagery of the traumatic event exhibited reduced autonomic responses to another exposure to the traumatic script a week later (Brunet, Orr, Tremblay, Robertson, Nader, & Pitman, 2008). These studies provide support for the idea that propranolol is somehow affecting the strength or salience of the emotional memory for the event to make it less traumatic. However, it is not "erasing" the memory as there are no reports that the participants in these studies have amnesia for their trauma. In addition, while propranolol is a centrally-acting antianxiety agent (Conant, Engler, Janowsky, Maisel, Gilpin, & LeWinter, 1989; Elman, Sugar, Fiscella, Deutsch, Noth, Nyberg, Packo, & Anderson, 1998; Muller, Mottweiler, & Bublak, 2005; Rodriguez-Romaguera, Sotres-Bayon, Mueller, & Quirk, 2009), administration of propranolol to PTSD patients not within the immediate post-stressor period or reactivation of the traumatic memory does not affect PTSD symptoms (McGhee, Maani, Garza, Desocio, Gaylord, & Black, 2009; Reist, Duffy, Fujimoto, & Cahill, 2001). Thus, it is the experience- or re-experience-paired administration of propranolol that alleviates symptoms of PTSD, presumably by reducing the emotional salience of the memory.

The findings of this dissertation suggest an interesting alternative interpretation of the effect of propranolol in PTSD patients. As noted above, PTSD patients given propranolol in an experience-paired manner are not amnesiac for their memory, but are less aroused, or stressed, by re-experiencing the memory. This experience may in fact reflect habituation of stress responses (e.g. autonomic responses in the above experiments) to the psychological stressor (the script-driven imagery of the traumatic event). In this interpretation, habituation to the stressor of their memory is impaired in PTSD patients, but not in the vast majority of people who experience trauma and are able to habituate normally. Importantly, PTSD is only diagnosed one month following the trauma, as experiencing PTSD-like symptoms for the first days and weeks following trauma is normal (Yehuda & LeDoux, 2007). This supports the idea that normal individuals are habituating over the first month, but PTSD patients fail to habituate. Thus,

it is possible that the effect of propranolol in PTSD patients is to *enhance* habituation. In this interpretation, propranolol damps β-AR activity to a level closer to that of nonpathological individuals. Thus, propranolol enhances the consolidation of the lack of threat posed by the traumatic memory, and therefore habituation, rather than blocking consolidation altogether. This idea could be explored by testing a dose-response curve for propranolol effects in PTSD patients after script-driven imagery in a manner similar to the methods used by Brunet and colleagues (see Figure 5.3). It may be that higher

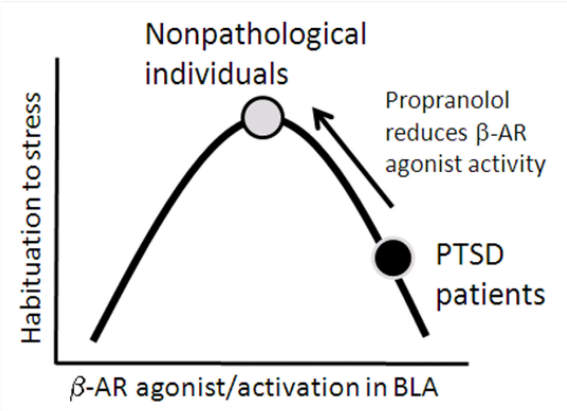

Figure 5.3 Model of the effects of propranolol administration in PTSD patients. Propranolol has been shown to reduce PTSD symptoms when given in conjunction with the trauma or reactivation of memory for the trauma, without causing amnesia for the trauma. PTSD can be thought of as a lack of habituation to the stressful re-experience of memories of the trauma. PTSD patients given propranolol may be better able to habituate as propranolol reduces β-AR agonist activity in the BLA, which may be hyperactive in these individuals as compared to nonpathological individuals exposed to trauma. However, increasing doses of propranolol should no longer be effective at reducing PTSD symptoms as it pushes individuals further left on the dose-response curve, reducing habituation. See text for details.

doses of propranolol are no longer beneficial at improving symptoms, as propranolol begins to prevent memory consolidation by blocking too many β-AR. Thus, PTSD patients may in part suffer from an inability to habituate due to hyperactivity at β–AR in the amygdala and other brain regions. Ultimately, individual differences in activity of stress-responsive systems, including individual differences in habituation to repeated stressor, may account for the incidence of stress-related psychological disorders such as PTSD.

Appendix A. Struggling behavior during restraint is regulated by stress experience

A.1 Introduction

Restraint elicits a variety of physiological stress responses and the magnitude of these can be decreased or increased by prior stress history. Repeated restraint exposure leads to decreases in hypothalamic-pituitary-adrenal (HPA) activation and fos mRNA expression in stress-responsive brain areas to the familiar restraint, a phenomenon known as habituation (Bhatnagar, Huber, Nowak, & Trotter, 2002; Girotti, Weinberg, & Spencer, 2007; Grissom, Iyer, Vining, & Bhatnagar, 2007; Jaferi & Bhatnagar, 2006). In contrast, in animals with a history of repeated experience with a different stressor, acute restraint can lead to facilitation, in which HPA and sympathetic activity meets or exceeds the responses induced by naive exposure to acute restraint (Bhatnagar & Dallman, 1998; Bhatnagar, Viau, Chu, Soriano, Meijer, & Dallman, 2000; Bhatnagar & Vining, 2003; Vining, Iyer, & Bhatnagar, 2007).

In addition to activating stress-sensitive physiological systems, acute restraint provokes a number of behaviors, including the production of fecal boli, ultrasonic vocalizations (Mitsushima, Yamada, Takase, Funabashi, & Kimura, 2006; Smriga & Torii, 2003) and struggling. In older literature, struggling during acute exposure to an immobilization paradigm was used to assess "irritability" during opiate withdrawal (Himmelsbach, Gerlach, & Stanton, 1935; Stanton, 1936). More recently, it was shown that strains of rats bred for high or low levels of amygdala excitability and seizure kindling exhibit high or low levels of struggling, respectively, during acute immobilization (Anisman, Lu, Song, Kent, McIntyre, & Merali, 1997; McIntyre, Kent, Hayley, Merali, & Anisman, 1999; Merali, Kent, Michaud, McIntyre, & Anisman, 2001). This difference in struggling paralleled between-strain differences in HPA responses to acute immobilization. Higher levels of struggling during acute immobilization are also associated with an increased incidence of gastric ulcers (Henke, 1990; Ushijima, Mizuki,

Hara, Kudo, Watanabe, & Yamada, 1986). This evidence suggests that the degree of struggling elicited by restraint or immobilization may itself be a useful measure of the stress response, and may parallel other indices of stress. However, the above studies focused only on acute exposure to restraint or immobilization and measurements of struggling were not well defined or quantified. If struggling to restraint does parallel other indices of the stress response in restrained animals, it should be predictably changed in magnitude depending on prior stress history. Therefore, the goal of the present studies was to measure struggling behavior and determine whether it is modified under conditions that typically produce habituated and facilitated HPA responses to restraint, and determine whether the degree of struggling observed follows the same pattern as HPA responses.

In the current experiments, using automated assessments of struggling, we were able to quantify these behaviors and found replicable effects of prior stress on restraint-induced struggling. We also observed similarities between these behaviors and HPA activity after 30 min of restraint. We analyzed behavior during acute restraint after exposure to repeated restraint (Experiments 1 and 3) or repeated swim (Experiments 2 and 4). In Experiment 1 we hypothesized that repeated restraint would lead to decreases in struggling during the 5th restraint, in parallel to HPA habituation seen with repeated restraint (Grissom, Kerr, & Bhatnagar, 2007). In Experiment 2 we hypothesized that prior exposure to repeated swim would lead to increases in struggling during heterotypic restraint, in similarly to HPA facilitation seen in this paradigm (Mercer, Grissom, & Bhatnagar, 2006). In Experiments 3 and 4, we examined the role of glucocorticoids in modulating behavioral habituation to repeated restraint (Experiment 3) and behavioral facilitation after repeated swim (Experiment 4) via adrenalectomy. Finally, we examined the effects of repeated restraint on behavior during forced swim (Experiment 5) to determine whether the increased struggling seen in facilitated responses to novel restraint is reflective of a general increase in activity upon exposure to a heterotypic stress. Overall, our findings indicate that struggling is a reproducible, reliable behavioral response which is measurable during restraint stress, follows a pattern similar to that seen in HPA activity, and is modified by prior stress history.

A.2 Methods

Animals

Male Sprague–Dawley rats (Charles Rivers) weighing between 225 and 250 g were individually housed in plastic tub cages with ad libitum access to food and water. The housing room was on a 12:12 l:d cycle with lights on at 06:00 h. Animals were given a 5–7 day acclimation period prior to the beginning of experimentation or surgery and were briefly handled during this period. All stress and experimentation took place between 08:00 and 12:00 h. All procedures were approved by the IACUC at the Children's Hospital of Philadelphia.

Stress paradigms

Restraint

Animals were placed in open-ended Plexiglas cylindrical restrainers measuring 6.7 cm in diameter and 22.3 cm in length and placed in a clean cage with bedding which held the restrainer in place. Restraint lasted for 30 min/day, at which point animals were returned to their home cage. Immediately after the last restraint exposure (day 5 or day 8, depending on the experiment) animals were decapitated and trunk blood collected for ACTH and corticosterone analysis.

Forced swim

Acute and repeated forced swim animals were placed in a glass chromatography jar (18 in. high × 8.75 in. outer diameter, Fisher Scientific, St. Louis, MO) filled two-thirds full of water measuring approximately 25 °C. Rats were swum for 15 min/day, a length of time allowing some comparability to the effects of 30 min stress while also being short enough for daily exposure to be tolerated. Animals given a single, acute forced swim exposure (Experiment 5) were decapitated immediately after swim and trunk blood was collected for analysis of ACTH and corticosterone.

Experimental design

Experiment 1: Acute restraint vs. repeated restraint

We hypothesized that the amount of struggling elicited by restraint would habituate over 5 days of repeated restraint. Rats were divided into two groups: the repeatedly restrained group was restrained for 30 min/day for 5 days, while the acute restraint group was undisturbed until day 5, at which point they were restrained as well. Video of 30 min restraint was obtained on day 5.

Experiment 2: Acute restraint only vs. acute restraint following repeated swim

In contrast to Experiment 1, we hypothesized that we would see facilitation in struggling during novel, heterotypic restraint on day 5 after 4 days of repeated forced swim compared to acute restraint alone. Rats were again divided into two groups: the repeated swim group was placed in a swim tank for 15 min/day for 4 days, while the acute group was undisturbed. On day 5 all animals were videotaped during 30 min restraint.

Experiment 3: Effects of adrenalectomy (ADX) on behavior during acute restraint vs. repeated restraint

Animals were either ADX or sham operated, as described below. After recovery, all animals were restrained for 8 days, during which video was captured on day 1 (acute response), and days 5 and 8 (habituated responses). We studied both days 5 and 8 to allow for comparisons to our previous studies(Grissom, Iyer, Vining, & Bhatnagar, 2007; Grissom, Kerr, & Bhatnagar, 2007).

Experiment 4: Effects of ADX on behavior during acute restraint vs. acute restraint following repeated swim

Animals were divided into a 2 × 2 design: ADX vs. sham, and repeated swim vs. no stress. After recovery from surgery, on days 1–4 all repeated swim animals were placed in a Porsolt tank for 15 min/day, while acute animals were undisturbed. On day 5 all animals were videotaped during 30 min restraint.

Experiment 5: Effects of repeated restraint on behavior during forced swim

It is possible that the increases in struggling seen during exposure to novel restraint after repeated swim in Experiments 2 and 4 reflect general increases in movement after repeated stress exposure. If this were so, one might expect an increase in movement during any novel stress after repeated exposure to a homotypic stress. We tested this hypothesis using two groups of animals: one group was repeatedly restrained for 7 days, while the other remained in the home cage. On day 8 all animals were videotaped during 15 min of heterotypic forced swim.

Adrenalectomy

In Experiments 3 and 4, which examined the effect of glucocorticoids on struggling behavior, all animals underwent bilateral surgical removal of the adrenals (ADX) or a sham surgery (the adrenals were exposed but not removed). ADX animals received 100 mg 35% corticosterone pellets subcutaneously, which provided a steady low dose of corticosterone at approximately the average daily value for intact rats(Akana, Chu, Soriano, & Dallman, 2001). Sham-operated animals received 100 mg pellets of cholesterol. ADX animals were also given 0.5% saline to drink for the duration of the experiment to prevent alterations in sodium balance that result from loss of adrenal hormones(Ohara, Cadnapaphornchai, Summer, Falk, Yang, Togawa, & Schrier, 2002). After surgery, animals were given between 5 and 7 days recovery before beginning experimentation. Completeness of adrenalectomies was verified by radioimmunoassay for corticosterone.

Video acquisition

Behavior during restraint

On test day video acquisition began by recording a background image that included the restrainers to subtract out of the final analysis. The camera used for acquisition (Panasonic WV-BP334), connected to an IBM ThinkCentre computer, was positioned and focused such that the restrainers were filmed from above and occupied as much of the screen width as possible. Videos were acquired directly onto the computer hard drive

as black/white MPEG-2 files with MediaCruise encoding software (Canopus, San Jose, CA).

Behavior during forced swim

As with restraint, video acquisition began by recording the filled tanks in position to obtain a background image. The camera was positioned such that four tanks, positioned side by side and filmed from the side, took up approximately 90% of the screen width. Videos were acquired as described above.

Behavioral analysis

Automatic coding of behavior was analyzed using the EthoVision Pro 3.1 video analysis software (Noldus Information Technology, Leesburg, VA) using the mobility parameter for analysis of behavior during both restraint and forced swim. Briefly, the software is able to give an index of an animal's mobility by detecting the extent of the animal as a field of pixels, and then assessing the percent pixel change between samples of the video. For all automated analysis the subtraction method of detection was used, detecting all objects different from background.

Automated analysis of restraint

Detection thresholds were set as to ensure that the head and body of the animals were included in observation but the tail was excluded. Thresholds of percent pixel change were set prior to any automated analysis of restraint. These thresholds were based on our preliminary observation of five restrained animals by an observer experienced in assessing behavior. Based on our observations, parameters were set in EthoVision to define three different levels of mobility: immobility, mobility (which we label as "light mobility" here to avoid confusion), and strong mobility. "Immobility" was visually indicated by an almost total lack of movement except for breathing was set to register between 0 and 2% pixel change. "Light mobility" was defined by smaller or slower movements of the head occurring throughout the 30 min restraint, including both sniffing and most bouts of grooming, corresponding to between 2 and 6% pixel change. The "strong mobility" parameter was defined by the largest pixel change percentages (greater than 6%, and generally not higher than 15%), which occurred during various

struggling/escape behaviors such as chewing on the restrainer, attempts to nose out, back out, or turn around in the restrainer, and rotation within the restrainer. The sampling rate was 5 times/s, allowing for fine-tuned distinctions of mobility. All of these parameters were set based on preliminary observations and before any experimentation was conducted. Data were analyzed both as 30 min totals and (in the case of strong mobility) in 5-min bins.

Correlation of manual coding with automated coding of behavior during restraint

As struggling behavior during restraint has not been well quantified previously, we assessed whether the strong mobility measurements obtained by analysis in EthoVision corresponded to observers' estimations of struggling behavior. For Experiment 1, two coders blind to experimental condition of the rats and familiar with the kinds of movements associated with struggling (chewing on the restrainer, attempts to nose out, back out, or turn around in the restrainer, and rotation within the restrainer) measured the total time spent struggling over the 30 min restraint for each animal in this experiment. Overall, these coders scores were very highly correlated with each other ($r(13) = 0.78$, $p \leq 0.005$) and the average of their scores were very highly correlated with the total time spent strongly mobile as assessed with EthoVision ($r\ (13) = 0.72$, $p \leq 0.01$), indicating that "strong mobility" is very closely associated with struggling as assessed by human observers. Furthermore, an unpaired *t*-test revealed significant differences between the acutely and repeatedly restrained rats in total time spent struggling over the 30 min restraint as assessed by human coders (acute restraint mean = 95.6 s, S.E.M. = 24.6; repeated restraint mean = 36.9 s, S.E.M. = 12.8; $t(13) = -2.193$, $p \leq 0.05$), paralleling the results seen in the automatic coding of strong mobility (see Section 3, Experiment 1).

Automated analysis of forced swim

The three mobility parameters (immobility, light mobility, strong mobility) were set to follow as closely as possible the distinctions commonly used to describe behavior during forced swim, as described below. To validate this method of measuring behavior during forced swim, a set of 8 naive animals separate from the experiments described here was

each swum for 5 min. During this time, video was simultaneously obtained of the side view (as in the current set of studies) and of the top-down view (as forced swim behavior is typically coded). Both the top-down and side views were hand-coded by an observer expert at coding forced swim behavior(Rittenhouse, Lopez-Rubalcava, Stanwood, & Lucki, 2002). These scores were converted to percent time spent immobile, swimming, or climbing/diving for comparison to the percent time spent immobile, lightly mobile, and strongly mobile, respectively, obtained by adjusting the percent pixel changes and sampling rate in EthoVision. Using these parameters immobility was indicated by less than 18.7% pixel change corresponding with a lack of movement other than that needed to keep the head afloat. Light mobility/swimming was indicated by 18.8–22.3% pixel change and corresponded to movements of the limbs associated with swimming, less severe than those associated with climbing. Strong mobility/climbing was indicated by greater than 22.3% pixel change and corresponded to large movements associated with attempts to escape the swim tank, including vigorous climbing near the sides of the tank and diving to the bottom of the tank. For swim videos, the sampling rate was averaged over 25 samples (1 averaged mobility score per 5 s), which minimized the influence of small variations of movement in the automatic analysis and produced scores similar to those obtained by hand coding. In addition to acquiring mobility data for analysis of Experiment 5, we also acquired and analyzed the distance moved within the tank via center-of-mass tracking, as this has been previously used as an inverse measure of immobility in the FST (Hedou, Pryce, Di Iorio, Heidbreder, & Feldon, 2001) and could be used to confirm results of the mobility analysis. All data were analyzed as 15 min totals and immobility, light mobility, and strong mobility were also analyzed in 5 min bins.

Hormone assays

At the end of 30 min restraint or 15 min swim, trunk blood was collected on ice into 15 ml conical tubes containing 100 µl sodium EDTA to prevent coagulation. Whole blood was centrifuged at 2500 rpm for 15 min. The plasma was reserved and frozen at −20 °C. Plasma ACTH and corticosterone were measured using kits from MP Biomedicals (Orangeburg, NY). The minimum levels of detection for ACTH and

corticosterone were 5.7 pg/ml and 0.6 μg/dl, respectively. Intra- and interassay variability was less than 10%.

Statistical analyses

Omnibus analyses of struggling behavior and hormones

All automated data, hand-coded behavioral data, and 30 min ACTH and corticosterone concentrations were analyzed with Statview software. For Experiments 1, 2, and 5, unpaired *t*-tests were conducted on total immobility, mobility, strong mobility or hormone concentrations, and in Experiment 5, total distance traveled was also analyzed. In Experiment 3 repeated measures ANOVA [Surgery (sham, ADX) × Day (1, 5, 8)] was conducted on total immobility, mobility, and strong mobility, and an unpaired *t*-test was used to compare hormone levels. In Experiment 4, 2 × 2 ANOVAs [Surgery (sham, ADX) × Stress (acute restraint, repeated swim followed by acute restraint)] were conducted on total immobility, mobility, strong mobility and hormone concentrations.

Timecourse analyses of struggling behavior

For Experiments 1–4, repeated measures ANOVAs were conducted on strong mobility measurements across 30 min restraint divided into 5 min timepoints, with timepoint as the repeated measure. For Experiment 1, this analysis was Stress (acute restraint, repeated restraint) × Timepoint (0–5, 5–10, 10–15, 15–20, 20–25, 25–30 min); Experiment 2, Stress (acute restraint, repeated swim followed by acute restraint) × Timepoint; Experiment 3, Surgery (sham, ADX) × Day of restraint (1, 5, 8) × Timepoint; Experiment 4, Surgery × Stress (acute restraint, repeated swim followed by acute restraint) × Timepoint. For Experiment 5 repeated measures ANOVA was conducted on measurements of all swimming behaviors (immobility, light mobility/swimming, and strong mobility/climbing) divided into 5 min timepoints, with timepoint as the repeated measure, making the analysis Stress (acute swim, repeated restraint followed by swim) × Timepoint (0–5, 5–10, 10–15 min). All significant effects were followed by Fisher's post hoc tests. The significance levels for all tests were set to $p \leq 0.05$.

A.3 Results

Experiment 1: Acute restraint vs. repeated restraint

Behavioral analyses

These data are presented in Figure A.1. We found a significant difference between acutely and repeatedly restrained rats in strong mobility over the 30 min restraint (t (13) = −2.1, $p \leq 0.05$). Acutely restrained rats spent more time strongly mobile than repeatedly restrained rats (Figure A.1a). Unpaired t-tests revealed no significant differences between acutely and repeatedly restrained rats in total time spent either immobile or lightly mobile (engaging in small movements not corresponding to struggling) during restraint on day 5.

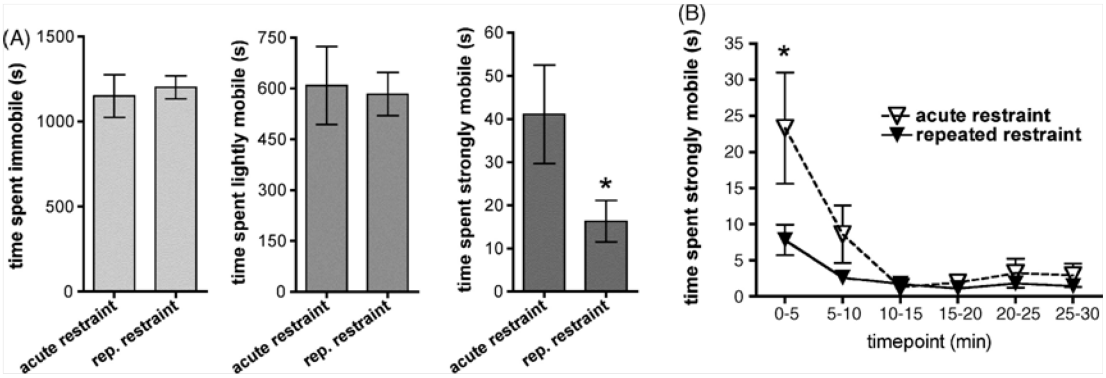

Figure A.1 In Experiment 1, animals were restrained on day 5 with (repeated restraint group) or without (acute restraint group) 4 prior days of repeated restraint stress. (A) Graphs show total time spent immobile, lightly mobile, and strongly mobile (struggling) during 30 min restraint on day 5. (B) Timecourse of time spent strongly mobile (struggling) across 30 min restraint on day 5. All data are expressed as mean ± S.E.M. Asterisks indicate repeated restraint group values significantly different from acute restraint group values.

We then analyzed time spent strongly mobile in 5 min increments to examine changes in strong mobility at different timepoints within the 30 min restraint period. Repeated measures ANOVA on Stress × Timepoint, with Timepoint as the repeated measure, revealed a significant Main effect of Stress (F (1,13) = 4.4, $p \leq 0.05$), a significant Main effect of Timepoint (F (5, 65) = 10.7, $p \leq 0.001$) and a significant Interaction effect (F (5, 65) = 3.2, $p \leq 0.01$). The significant Main effect of Stress indicated that repeatedly restrained rats showed lower levels of struggling overall than the acutely restrained rats. The significant main effect of time indicated that in all animals, struggling was highest

during the first 5 min (0–5 min) of restraint than at any other time. Fisher's post hoc analyses of the significant interaction test revealed that acutely restrained animals spent significantly more time strongly mobile during the first 5 min than repeatedly restrained rats at all timepoints and acutely stressed rats at any other timepoints.

HPA response

ACTH concentrations at the end of 30 min restraint were significantly reduced in repeatedly restrained rats as compared to acutely restrained rats (t (13) = −2.1, $p \leq 0.05$; Table A.1). Corticosterone levels at 30 min were not different between groups at this timepoint.

Experiment 2: Acute restraint with or without prior repeated forced swim exposure

Behavioral responses

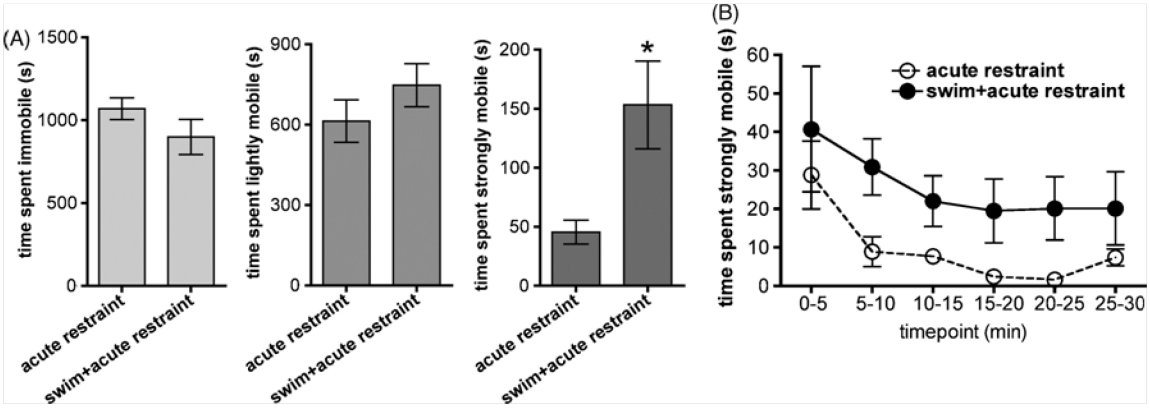

Figure A.2 In Experiment 2, animals were restrained on day 5 after 4 days of repeated forced swim (swim + acute restraint group) or without prior swim (acute restraint group). (A) Graphs show total time spent immobile, lightly mobile, and strongly mobile (struggling) during 30 min restraint on day 5 in animals. (B) Timecourse of time spent strongly mobile (struggling) across 30 min restraint on day 5. All data are expressed as mean ± S.E.M. Asterisk indicates that acutely restrained rats exhibited higher strong mobility than acutely restrained rats after swim throughout the 30 min period of testing.

These data are presented in Figure A.2. Repeatedly swum animals spent significantly more total time strongly mobile during novel restraint than acutely restrained rats (t(20) = −2.6, $p \leq 0.01$). No differences were seen between repeatedly swum animals in novel restraint and acutely restrained rats in total time spent immobile or lightly mobile during restraint.

Repeated measures ANOVA on Stress × Timepoint revealed a Main effect of Stress (F (1, 20) = 7.0, $p \leq 0.01$) and a Main effect of Timepoint (F (5, 100) = 4.2, $p \leq 0.001$) but no Interaction effect. The Main effect of Stress indicated that overall, repeatedly swum animals spent more time strongly mobile during novel restraint than acutely restrained animals. Fisher's post hoc analyses of the significant Main effect of Timepoint indicated that strong mobility in the first five minutes was significantly higher than at any other time points (independent of acute or repeated stressed groups).

HPA responses

ACTH and corticosterone levels at the end of 30 min restraint were similar between animals that were experiencing acute restraint and animals that had previously experienced repeated swim (Table A.1).

Experiment 3: Acute vs. repeated restraint in adrenalectomized animals

Behavioral responses

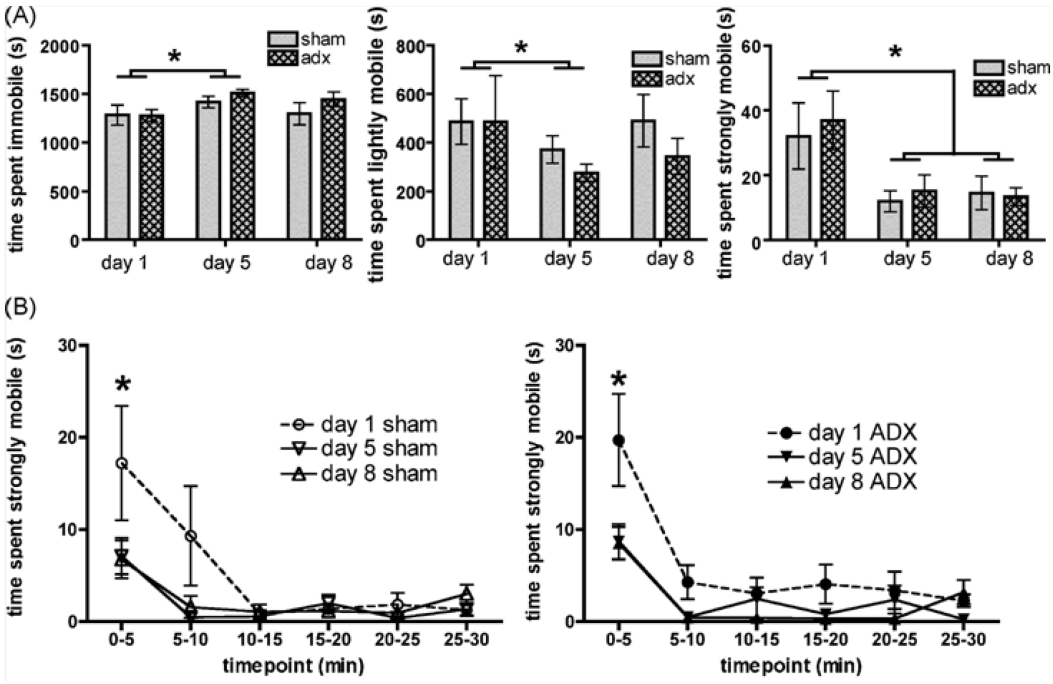

Figure A.3 In Experiment A.3 animals were first sham operated (sham) or adrenalectomized (ADX) and replaced with subcutaneous corticosterone pellets prior to 8 days repeated restraint. (A) Graphs show total time spent immobile, lightly mobile, and strongly mobile (struggling) in both groups on days 1 (acute restraint), 5, and 8 of restraint. (B) Timecourse of time spent strongly mobile (struggling) across 30 min restraint on days 1, 5, and 8, divided by surgery. All data are expressed as mean ± S.E.M. Asterisks indicate repeated restraint group values significantly different from overall acute restraint group values.

Repeated measures ANOVAs (Surgery × Day) were conducted for total time spent immobile, lightly mobile, and strongly mobile (Figure A.3a). Importantly, total time spent strongly mobile showed no effect of Surgery, but a significant Main effect of Day (F (2, 32) = 10.5, $p \leq 0.001$) showing in all animals a significant decrease in strong mobility between days 1 and 5, and between days 1 and 8, and no difference between days 5 and 8. In immobility, there was a significant Main effect of Day (F (2, 32) = 4.9, $p \leq 0.01$), such that total immobility significantly increased between days 1 and 5, but no difference was observed between days 5 and 8 or between days 1 and 8. Changes in light mobility between days showed a similar pattern, revealing a significant Main effect of Day (F (2, 32) = 4.2, $p \leq 0.05$) showing a significant decrease in time spent lightly mobile between days 1 and 5, and no difference between days 5 and 8 or days 1 and 8. No effect of Surgery was found in any analysis.

Repeated measures ANOVA (Surgery × Day of restraint × Timepoint) on time spent strongly mobile each day divided into 5 min increments revealed a significant Main effect of Day (F (2, 32) = 10.5, $p \leq 0.001$) indicating that strong mobility was significantly decreased from day 1 to 5, and 1 to 8, with no difference between days 5 and 8. A significant Main effect of Timepoint (F (5, 80) = 18.2, $p \leq 0.001$) was also seen, indicating that strong mobility was greater during the first 5 min of restraint than any other time period, regardless of the day or surgical treatment. Finally, a significant Day × Timepoint Interaction (F (10, 160) = 5.4, $p \leq 0.001$) was seen. Post hoc analyses indicated that both sham and ADX animals showed greater levels of strong mobility in the first 5 min on day 1 than on days 5 and 8. There were no other significant effects.

HPA responses

Blood plasma samples were taken at the end of stress on day 8 to confirm ADX (Table A.1). Unpaired t-tests between sham operated and ADX animals after 8 days repeated restraint showed a significant difference in ACTH ($t(16) = -13.4$, $p \leq 0.001$) and corticosterone levels ($t(16) = 5.3$, $p \leq 0.001$) between groups. ADX animals showed significantly increased ACTH concentration, consistent with a lack of corticosterone negative feedback, and significantly lower corticosterone levels consistent with corticosterone replacement via the subcutaneous pellets, compared to sham-adrenalectomized animals.

Experiment 4: Acute restraint with or without prior swim exposure in adrenalectomized animals

Behavioral responses

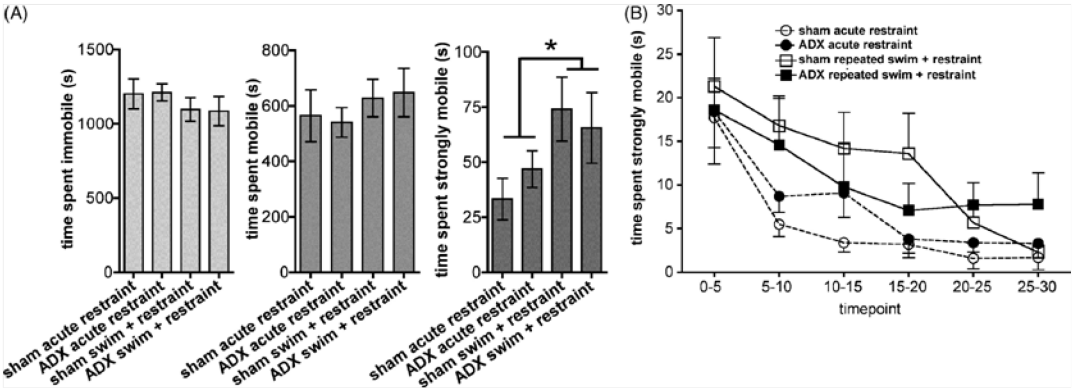

Figure A.4 In Experiment A.4 animals were first sham operated (sham) or adrenalectomized (ADX) and replaced with subcutaneous corticosterone pellets, then were either undisturbed until restraint on day 5 (acute restraint) or given 4 days of repeated forced swim prior to restraint on day 5 (swim + acute restraint). (A) Graphs show total time spent immobile, lightly mobile, and strongly mobile (struggling) in sham and ADX animals during 30 min restraint on day 5. (B) Timecourse of time spent strongly mobile (struggling) across 30 min restraint on day 5, divided by both stress history and by surgery. All data are expressed as mean ± S.E.M. Asterisks indicate overall repeated swim + restraint group values significantly different from overall acute restraint values.

These results are shown in Fig. A.4. A 2 × 2 ANOVA conducted on total time spent strongly mobile on day 5 showed a significant Main effect of Stress (F (1, 27) = 6.5, $p \leq 0.01$), indicating that animals exposed to repeated forced swim prior to restraint on day 5 spent more total time strongly mobile than acutely restrained animals. 2 × 2 ANOVA (Surgery × Stress) conducted on total time spent immobile or lightly mobile during restraint on day 5 showed no significant effects. No effect of Surgery and no Interaction effect were observed in any analysis.

Repeated measures ANOVA (Surgery × Stress × Timepoint) were conducted on time spent strongly mobile on day 5 divided into 5 min timepoints. A Main effect of Stress (F (1, 28) = 5.8, $p \leq 0.05$), indicated that, overall, animals which received repeated swim prior to restraint showed significantly elevated levels of strong mobility as compared to acutely restrained animals, regardless of surgery (comparison not specifically shown). A Main effect of Timepoint during restraint (F (5, 140) = 16.3, $p \leq 0.001$) was also seen, indicating that strong mobility was higher during the first 5 min than at all other

timepoints, higher at 5–10 min than at any time in the last 15 min of restraint, and higher at 10–15 min than at any time in the last 10 min of restraint. No effects of Surgery and no significant Interactions were seen. Therefore, animals exposed to repeated swim + restraint exhibited higher strong mobility over the 30 min period of restraint compared to animals exposed to restraint alone. Adrenalectomy did not significantly alter this finding.

HPA responses

A 2 × 2 ANOVA (Surgery × Stress) conducted on ACTH levels at the end of 30 min restraint on day 5 showed no Main effect of Stress, but a significant Main effect of Surgery (F (1, 27) = 97.9, $p \leq 0.001$) indicating that the ACTH levels of ADX animals were significantly elevated, regardless of stress history. No Interaction effects were seen.

	Condition	ACTH	Corticosterone
Experiment A.1	Acute restraint	177.0 ± 48.4	19.1 ± 5.0
	Repeated restraint	70.6 ± 21.9*	21.7 ± 9.3
Experiment A.2	Acute restraint	194.5 ± 23.7	25.5 ± 2.9
	Repeated swim + acute restraint	209.2 ± 32.7	23.5 ± 2.2
Experiment A.3	SHAM + repeated restraint	98.8 ± 17.8	16.4 ± 2.9
	ADX + repeated restraint	1594.5 ± 87.1†	3.9 ± 0.4†
Experiment A.4	Sham + acute restraint	201.1 ± 99.5	14.7 ± 2.1
	Sham + repeated swim + acute restraint	211.3 ± 49.7	26.4 ± 3.0*
	ADX + acute restraint	1086.9 ± 134.6†	2.4 ± 0.3†
	ADX + repeated swim + acute restraint	1383.5 ± 85.8†	2.2 ± 0.3†

Table A.1 Plasma ACTH and corticosterone levels collected at the end of 30 min restraint on day 5 (Experiments 1, 2, and 4) or day 8 (Experiment 3). In Experiments 1 and 2, animals were acutely restrained on day 5 with or without 4 days prior repeated restraint (Experiment 1) or repeated swim (Experiment 2). In Experiments 3 and 4, animals were either sham operated or adrenalectomized (ADX) prior to repeated stress, and blood samples were taken at the end of restraint on day 8 or day 5 to confirm ADX. All data are expressed as mean ± S.E.M. Asterisks (*) indicate repeated stress group values are significantly different than comparable acute restraint group values. Crosses (†) indicate ADX values are significantly different than comparable sham group values.

2 × 2 ANOVA (Surgery × Stress) on corticosterone levels at the end of 30 min restraint showed significant Main effects of Stress (F (1, 27) = 4.3, $p \leq 0.05$), indicating that overall, plasma corticosterone concentrations were significantly higher in repeatedly swum animals in acute restraint than naive animals in acute restraint. There was also a Main effect of Surgery (F (1, 27) = 90.5, $p \leq 0.001$), indicating that the sham-operated animals had significantly higher corticosterone levels than ADX animals overall. A significant Interaction effect (F (1, 27) = 4.8, $p \leq 0.05$) was also seen, indicating that the increase in corticosterone due to prior swim stress was only significant in the sham operated group.

Experiment 5: Forced swim exposure in naive vs. repeatedly restrained animals

Behavioral analyses

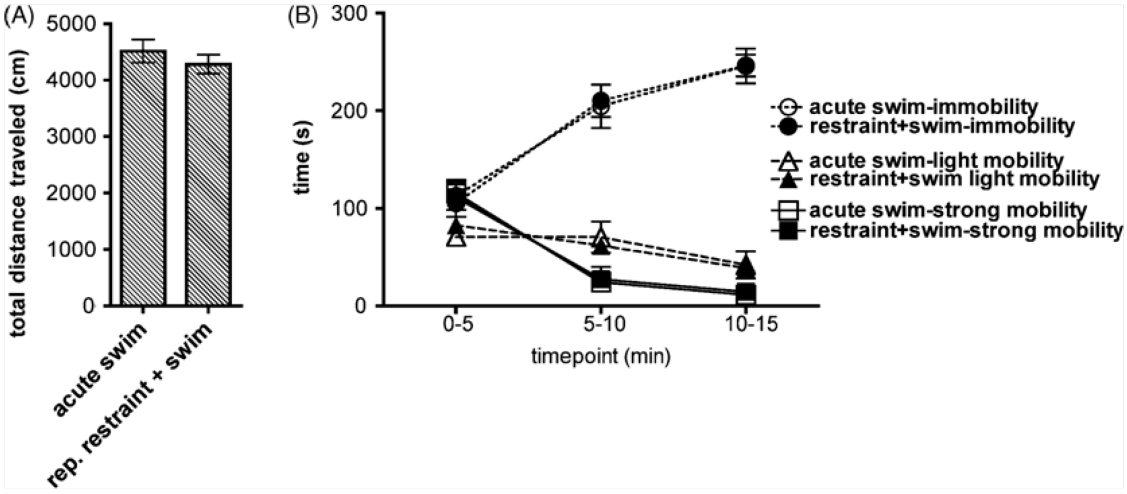

Figure A.5 (A) In Experiment 5, animals were placed in forced swim for 15 min with (rep. restraint + swim group) or without (acute swim group) 7 prior days of repeated restraint. (A) Graphs show total distance traveled during 15 min forced swim on day 8. (B) Timecourse of times spent immobile, lightly mobile, and strongly mobile across 15 min swim on day 8. All data are expressed as mean ± S.E.M.

These data are presented in Figure A.5. No differences were seen between animals receiving acute exposure to 15 min swim, compared to animals receiving 15 min swim after 7 previous days of 30 min restraint, in time spent immobile, lightly mobile (corresponding to swimming behavior), or strongly mobile (corresponding to climbing behavior) over the total 15 min (not shown).

Following the methods of Hedou et al. (2001), we also examined total distance traveled via center of mass tracking, which was verified previously as an inverse measure of immobility in the FST. An unpaired *t*-test comparing total distance traveled in acutely swum rats vs. rats exposed to repeated restraint prior to acute swim revealed no differences between groups. We examined the correlation between total distance traveled and total immobility, light mobility, and strong mobility and found a negative correlation between immobility and total distance traveled ($r(19) = -0.46$, $p \leq 0.05$) and a corresponding positive correlation between strong mobility and total distance traveled ($r(19) = 0.48$, $p \leq 0.05$).

Repeated measures ANOVAs (Stress × Timepoint) were conducted on time spent immobile, mobile, and strongly mobile divided in 5 min increments. A significant Main effect of Timepoint in all three analyses was observed, such that immobility increased over the 15 min period in all animals (F (2, 36) = 120.1, $p \leq 0.001$) and mobility/swimming and strong mobility/climbing decreased in all animals (F (2, 36) = 13.2, $p \leq 0.001$ and F (2, 36) = 120.7, $p \leq 0.001$, respectively). Post hoc analyses indicate that immobility significantly increased across all three timepoints, light mobility was significantly decreased at the 10–15 min timepoint compared to the first and second 5 min, and strong mobility was significantly higher in the first 5 min than the remainder of the swim. No effects of Stress and no Interactions were seen.

A.4 Discussion

The experiments presented here indicate that struggling during restraint is a stress-induced behavior that is consistent and readily quantifiable. Struggling can be modified by prior stress history in a manner similar to the HPA response, but does not seem to be regulated by stress-induced increases in circulating glucocorticoids. In Experiment A.1, we found that in comparison with acute restraint, repeated exposure to restraint significantly reduced the amount of restraint-elicited struggling. Likewise, in Experiment A.2, animals that were repeatedly swum struggled significantly more during acute restraint than naive animals in acute restraint, demonstrating behavioral facilitation (Figure A.2). In both of these experiments, acutely restrained animals displayed a stereotypical struggling response to restraint, with levels of struggling highest during the

first 5 min of restraint and showing rapid within-restraint habituation. Thus the reduction of struggling in the habituated animals in Experiment A.1 is most clearly visible during the first 5 min of restraint, after which point all animals largely stopped struggling. In contrast, the facilitation of struggling in Experiment A.2 is significant only after the first 5 min of restraint, when the facilitated animals continued to struggle at a point at which acutely restrained animals no longer struggled.

For the most part, HPA responses followed what was expected in terms of habituation to homotypic stress and facilitation to heterotypic stress exposure, a pattern also observed in the behavioral struggling responses. The behavioral habituation in Experiment A.1 was associated with habituation of ACTH, though corticosterone did not habituate (Table A.1). The most likely reason is that we only collected one blood sample at 30 min and it is possible that corticosterone habituated in these animals at a time following termination of restraint. The possibility remains that changes in adrenal responsivity or intra-adrenal mechanisms may have prevented habituation at the adrenal level, though many studies have observed habituation of both ACTH and corticosterone (Bhatnagar, Huber, Nowak, & Trotter, 2002; Girotti, Weinberg, & Spencer, 2007; Grissom, Iyer, Vining, & Bhatnagar, 2007; Jaferi & Bhatnagar, 2006). In animals exposed to restraint after repeated swim in Experiment 2, ACTH and corticosterone levels are similar to those of acutely restrained rats. Therefore, HPA activity in repeated swim rats is consistent with facilitation, as defined by Dallman and Jones(Dallman & Jones, 1973), as their HPA responses match those of acutely stressed. We have previously observed facilitation of HPA responses to restraint after repeated swim (Mercer, Grissom, & Bhatnagar, 2006).

As discussed in the introduction, struggling in rats during acute immobilization has been measured in a few studies, but this is the first detailed description of the different behaviors that we collectively classify as “struggling.” These behaviors include chewing on the restrainer, attempts to nose out, back out, or turn around in the restrainer, and rotation within the restrainer. The use of automated analysis software in our experiments greatly streamlined our analysis of struggling behavior in restrained rats. Nevertheless, the software is unable to note distinctions between the different sorts of large movements that might trigger “strong mobility”/struggling. Therefore, we do not know whether in a

given experiment one group spent more time chewing, or another spent more time turning around and these behaviors could reflect very different states of the animal. Future investigation of struggling behavior may find analysis of these individual behaviors informative.

A number of behavioral tasks currently exist which can be used to assess anxiety-like or depressive-like behaviors in an animal. However, the experiments presented here do not address whether one or more psychological states are reflected by increased or decreased struggling behavior. It has been previously shown that levels of struggling during immobilization are decreased by peripheral administration of GABA agonists (Ushijima, Mizuki, Hara, Kudo, Watanabe, & Yamada, 1986) and morphine(Tanaka, Kohno, Tsuda, Nakagawa, Ida, Iimori, Hoaki, & Nagasaki, 1983), and increased by naloxone(Tanaka, Kohno, Tsuda, Nakagawa, Ida, Iimori, Hoaki, & Nagasaki, 1983). Based on these data, it is possible that struggling may reflect an anxiety-like state. If a relationship between this behavior and an anxiety or depressive-like state is found, struggling could be used to provide a measure of an animal's behavioral state while permitting simultaneous analysis of peripheral or central physiological markers in response to uninterrupted restraint. This potential is especially pertinent to designs involving habituation to repeated restraint or facilitation to novel restraint. For instance, while in these studies we did not wish to jeopardize novel behavioral data to obtain multiple blood samples, our results indicate that significant differences in struggling are observed within the first 15 min of restraint. Subsequent experiments using this measure could potentially obtain blood samples repeatedly after the first 15 min of restraint, allowing more direct comparisons of behavioral measures with measures such as HPA activity and mRNA expression in specific stress-regulatory brain regions.

Given the similarities between struggling behavior and HPA activity seen in Experiments A.1 and A.2, it was possible that struggling behavior is regulated by stress-induced increases in circulating glucocorticoids. In Experiments A.3 and A.4, we examined the effects of eliminating the corticosterone response to stress on the generation of acute, habituated, or facilitated struggling responses to restraint. Adrenalectomy with corticosterone replacement prior to the beginning of repeated restraint or swim had no

statistically significant effects on the magnitude of struggling in acutely restrained animals, or on the development of behavioral habituation or facilitation. We conclude that stress-induced increases in glucocorticoids do not regulate struggling to restraint. However, additional study may be required before we can be sure of this interpretation. In Experiment A.3, the struggling response habituated over repeated restraint, over the course of which corticosterone release would progressively diminish even in sham operated animals. In contrast, in Experiment A.4 struggling was expected to increase in animals exposed to restraint after repeated swim. While the statistical analyses did not reveal significant effects of ADX on behavioral facilitation, the graphs appear to indicate that facilitation in struggling was somewhat blunted in ADX animals restrained after repeated swim compared to sham animals. It is possible that in this experiment, the corticosterone replacement was insufficient to allow behavioral facilitation to the same level as the sham operated animals. A future study could test this idea by examining behavioral facilitation in ADX animals with varying levels of corticosterone replacement. No corticosterone replacement may abolish behavioral facilitation, while higher levels may be required for behavioral facilitation to proceed normally. At present, however, our results indicate that (1) struggling during restraint is influenced by stress history, but (2) this modulation does not appear to be regulated by stress-induced increases in glucocorticoids.

The results of Experiments A.2 and A.4, showing an increase in struggling to restraint after repeated swim, raise the question of whether simple exposure to any heterotypic stressor after a period of repeated homotypic stress increases movement in general, or whether the facilitation of struggling seen here is specific to restraint. In Experiment A.5 we tested this hypothesis by examining behavior during forced swim with or without 7 days of previous repeated restraint, and found no effect on immobility, light mobility, or strong mobility (mapping onto immobility, swimming, and climbing, respectively) during forced swim. We also analyzed total distance traveled, which is a previously validated index of forced swim behavior (Hedou, Pryce, Di Iorio, Heidbreder, & Feldon, 2001) and also found no difference between groups. However, total distance traveled was found to be significantly negatively correlated with immobility and significantly positively correlated with strong mobility, a finding similar to previous work using total distance

traveled (Hedou, Pryce, Di Iorio, Heidbreder, & Feldon, 2001). Overall, these results do not support the idea that movement is increased generally in response to a heterotypic stressor after a period of homotypic stress. There are of course several important caveats to this interpretation of the above experiment. First, repeated restraint is not as severe a stressor as repeated forced swim (Dal-Zotto, Marti, & Armario, 2000; Rittenhouse, Lopez-Rubalcava, Stanwood, & Lucki, 2002). It is possible that using a stronger repeated stressor might have elicited differences in overall mobility during swim in Experiment A.5. Second, the test swim in Experiment A.5 may have required more movement in general than the test restraints in Experiments A.2 and A.4. Animals cannot remain truly immobile during forced swim in the same way as is possible during restraint, and as a result movement during swim might be high enough in general to obscure group differences. Regardless, these caveats do not contradict the idea that struggling during restraint is a behavior distinct from the behaviors seen during forced swim and does not reflect a non-specific increase in motor activity after repeated stress.

It is possible that struggling during restraint is stimulated by some of the same circuitry that stimulates the HPA axis, which may account for the similarity between the behavioral and hormonal responses. While the present studies do not address what brain areas may be involved in struggling during acute restraint or the habituation or facilitation of this behavior, other literature has shown an interesting relationship between amygdala activity and struggling during acute immobilization. Strain differences in amygdala excitability, which result in either high or low propensity towards seizure kindling, lead respectively to high or low HPA activity and struggling during acute immobilization (Anisman, Lu, Song, Kent, McIntyre, & Merali, 1997; McIntyre, Kent, Hayley, Merali, & Anisman, 1999; Merali, Kent, Michaud, McIntyre, & Anisman, 2001). Additionally, induction of dentate gyrus LTP via basolateral amygdala stimulation leads to decreases in struggling during acute immobilization (Henke, 1990). It is possible that the co-regulation of HPA and behavioral responses may be related to the functioning of one or more amygdalar nuclei and potentially other limbic structures, but this remains a question to be addressed by future studies.

The studies presented here demonstrate that struggling during restraint can be bidirectionally modified by prior stress history, habituating and facilitating in parallel with HPA activity in response to restraint. There are likely a number of significant relationships that remain to be found between struggling and other physiological and neural changes induced by restraint. There is already a literature indicating that greater amounts of struggling during immobilization is associated with altered immune system functioning (Anisman, Lu, Song, Kent, McIntyre, & Merali, 1997), increased severity of gastric ulcers (Henke, 1990; Ushijima, Mizuki, Hara, Kudo, Watanabe, & Yamada, 1986), increased lactate levels in blood (Rand, Kinnaird, Baglioni, Blackshaw, & Priest, 2002) and lactic acid in muscle indicative of hyperglycemia and metabolic acidosis (Bush, Custer, Smeller, & Bush, 1977). In these studies the amount of struggling observed was directly related to the severity of negative stress-induced physiological outcomes. These physiological changes, induced by acute or repeated stress and associated with struggling, may habituate and facilitate along with struggling, making it a potentially important and useful behavioral index of coping in response to restraint.

References

Aguilera, G., Subburaju, S., Young, S., & Chen, J. (2008). The parvocellular vasopressinergic system and responsiveness of the hypothalamic pituitary adrenal axis during chronic stress. *Progress in brain research, 170*

Akana, S. F., Chu, A., Soriano, L., & Dallman, M. F. (2001). Corticosterone exerts site-specific and state-dependent effects in prefrontal cortex and amygdala on regulation of adrenocorticotropic hormone, insulin and fat depots. *Journal of neuroendocrinology, 13*(7), 625-637.

Akirav, I., & Richter-Levin, G. (2006). Factors that determine the non-linear amygdala influence on hippocampus-dependent memory. *Dose-response : a publication of International Hormesis Society, 4*(1), 22-37.

Allaman, I., Papp, M., Kraftsik, R., Fiumelli, H., Magistretti, P. J., & Martin, J. L. (2008). Expression of brain-derived neurotrophic factor is not modulated by chronic mild stress in the rat hippocampus and amygdala. *Pharmacological reports : PR, 60*(6), 1001-1007.

Anisman, H., Lu, Z. W., Song, C., Kent, P., McIntyre, D. C., & Merali, Z. (1997). Influence of psychogenic and neurogenic stressors on endocrine and immune activity: Differential effects in fast and slow seizing rat strains. *Brain, behavior, and immunity, 11*(1), 63-74.

Armario, A. (2006). The hypothalamic-pituitary-adrenal axis: What can it tell us about stressors? *CNS & neurological disorders drug targets, 5*(5), 485-501.

Armario, A., Castellanos, J. M., & Balasch, J. (1984). Adaptation of anterior pituitary hormones to chronic noise stress in male rats. *Behavioral and neural biology, 41*(1), 71-76.

Armario, A., Hidalgo, J., & Giralt, M. (1988). Evidence that the pituitary-adrenal axis does not cross-adapt to stressors: Comparison to other physiological variables. *Neuroendocrinology, 47*(3), 263-267.

Armario, A., Lopez-Calderon, A., Jolin, T., & Balasch, J. (1986). Response of anterior pituitary hormones to chronic stress. the specificity of adaptation. *Neuroscience and biobehavioral reviews, 10*(3), 245-250.

Armario, A., Restrepo, C., Castellanos, J., & Balasch, J. (1985). Dissociation between adrenocorticotropin and corticosterone responses to restraint after previous chronic exposure to stress. *Life Sciences, 36*(22)

Armario, A., Valles, A., Dal-Zotto, S., Marquez, C., & Belda, X. (2004). A single exposure to severe stressors causes long-term desensitisation of the physiological response to the homotypic stressor. *Stress (Amsterdam, Netherlands), 7*(3), 157-172.

Arnhold, M. M., Wotus, C., & Engeland, W. C. (2007). Differential regulation of parvocellular neuronal activity in the paraventricular nucleus of the hypothalamus following single vs. repeated episodes of water restriction-induced drinking. *Experimental neurology, 206*(1), 126-136.

Aston-Jones, G., Chiang, C., & Alexinsky, T. (1991). Discharge of noradrenergic locus coeruleus neurons in behaving rats and monkeys suggests a role in vigilance. *Progress in brain research, 88*, 501-520.

Aubry, J. M., Bartanusz, V., Jezova, D., Belin, D., & Kiss, J. Z. (1999). Single stress induces long-lasting elevations in vasopressin mRNA levels in CRF hypophysiotrophic neurones, but repeated stress is required to modify AVP immunoreactivity. *Journal of neuroendocrinology, 11*(5), 377-384.

Balu, D. T., Hoshaw, B. A., Malberg, J. E., Rosenzweig-Lipson, S., Schechter, L. E., & Lucki, I. (2008). Differential regulation of central BDNF protein levels by antidepressant and non-antidepressant drug treatments. *Brain research, 1211*, 37-43.

Bassett, J. R., Cairncross, K. D., & King, M. G. (1973). Parameters of novelty, shock predictability and response contigency in corticosterone release in the rat. *Physiology & Behavior, 10*(5), 901-907.

Bergström, A., Jayatissa, M., Mørk, A., & Wiborg, O. (2008). Stress sensitivity and resilience in the chronic mild stress rat model of depression; an in situ hybridization study. *Brain research, 1196*

Bhatnagar, S., & Dallman, M. (1998). Neuroanatomical basis for facilitation of hypothalamic-pituitary-adrenal responses to a novel stressor after chronic stress. *Neuroscience, 84*(4)

Bhatnagar, S., Huber, R., Nowak, N., & Trotter, P. (2002). Lesions of the posterior paraventricular thalamus block habituation of hypothalamic-pituitary-adrenal responses to repeated restraint. *Journal of neuroendocrinology, 14*(5)

Bhatnagar, S., & Meaney, M. J. (1995). Hypothalamic-pituitary-adrenal function in chronic intermittently cold-stressed neonatally handled and non handled rats. *Journal of neuroendocrinology, 7*(2), 97-108.

Bhatnagar, S., Mitchell, J. B., Betito, K., Boksa, P., & Meaney, M. J. (1995). Effects of chronic intermittent cold stress on pituitary adrenocortical and sympathetic adrenomedullary functioning. *Physiology & Behavior, 57*(4), 633-639.

Bhatnagar, S., Viau, V., Chu, A., Soriano, L., Meijer, O. C., & Dallman, M. F. (2000). A cholecystokinin-mediated pathway to the paraventricular thalamus is recruited in chronically stressed rats and regulates hypothalamic-pituitary-adrenal function. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 20*(14), 5564-5573.

Bhatnagar, S., & Vining, C. (2003). Facilitation of hypothalamic-pituitary-adrenal responses to novel stress following repeated social stress using the resident/intruder paradigm. *Hormones and behavior, 43*(1)

Bhatnagar, S., Vining, C., & Denski, K. (2004). Regulation of chronic stress-induced changes in hypothalamic-pituitary-adrenal activity by the basolateral amygdala. *Annals of the New York Academy of Sciences, 1032*

Bonini, J., Cammarota, M., Kerr, D., Bevilaqua, L., & Izquierdo, I. (2005). Inhibition of PKC in basolateral amygdala and posterior parietal cortex impairs consolidation of inhibitory avoidance memory. *Pharmacology, biochemistry, and behavior, 80*(1)

Borrell, J., Torrellas, A., Guaza, C., & Borrell, S. (1980). Sound stimulation and its effects on the pituitary-adrenocortical function and brain catecholamines in rats. *Neuroendocrinology, 31*(1), 53-59.

Botreau, F., & Gisquet-Verrier, P. (2006). Memory reactivation, dissociated from behavioural expression, decreases ERK phosphorylation in the rat prefrontal cortex and amygdala. *Behavioural brain research, 169*(1), 176-180.

Bowers, S. L., Bilbo, S. D., Dhabhar, F. S., & Nelson, R. J. (2008). Stressor-specific alterations in corticosterone and immune responses in mice. *Brain, behavior, and immunity, 22*(1), 105-113.

Breslau, N., & Kessler, R. C. (2001). The stressor criterion in DSM-IV posttraumatic stress disorder: An empirical investigation. *Biological psychiatry, 50*(9), 699-704.

Brunet, A., Orr, S. P., Tremblay, J., Robertson, K., Nader, K., & Pitman, R. K. (2008). Effect of post-retrieval propranolol on psychophysiologic responding during subsequent script-driven traumatic imagery in post-traumatic stress disorder. *Journal of psychiatric research, 42*(6), 503-506.

Buffalari, D. M., & Grace, A. A. (2007). Noradrenergic modulation of basolateral amygdala neuronal activity: Opposing influences of alpha-2 and beta receptor activation. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 27*(45), 12358-12366.

Buffalari, D. M., & Grace, A. A. (2009). Chronic cold stress increases excitatory effects of norepinephrine on spontaneous and evoked activity of basolateral amygdala neurons. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP), 12*(1), 95-107.

Bush, M., Custer, R., Smeller, J., & Bush, L. M. (1977). Physiologic measures of nonhuman primates during physical restraint and chemical immobilization. *Journal of the American Veterinary Medical Association, 171*(9), 866-869.

Campeau, S., Falls, W. A., Cullinan, W. E., Helmreich, D. L., Davis, M., & Watson, S. J. (1997). Elicitation and reduction of fear: Behavioural and neuroendocrine indices and brain induction of the immediate-early gene c-fos. *Neuroscience, 78*(4), 1087-1104.

Carter, R. N., Pinnock, S. B., & Herbert, J. (2004). Does the amygdala modulate adaptation to repeated stress? *Neuroscience, 126*(1), 9-19.

Castillo, D. V., Figueroa-Guzman, Y., & Escobar, M. L. (2006). Brain-derived neurotrophic factor enhances conditioned taste aversion retention. *Brain research, 1067*(1), 250-255.

Cecchi, M., Khoshbouei, H., & Morilak, D. A. (2002). Modulatory effects of norepinephrine, acting on alpha 1 receptors in the central nucleus of the amygdala, on behavioral and neuroendocrine responses to acute immobilization stress. *Neuropharmacology, 43*(7), 1139-1147.

Chandramohan, Y., Droste, S., Arthur, J., & Reul, J. (2008). The forced swimming-induced behavioural immobility response involves histone H3 phospho-acetylation and c-fos induction in dentate gyrus granule neurons via activation of the N-methyl-D-aspartate/extracellular signal-regulated kinase/mitogen- and stress-a. *The European journal of neuroscience, 27*(10)

Chwang, W., Arthur, J., Schumacher, A., & Sweatt, J. (2007). The nuclear kinase mitogen- and stress-activated protein kinase 1 regulates hippocampal chromatin remodeling in memory formation. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 27*(46)

Chwang, W., O'Riordan, K., Levenson, J., & Sweatt, J. (2006). ERK/MAPK regulates hippocampal histone phosphorylation following contextual fear conditioning. *Learning & memory (Cold Spring Harbor, N.Y.), 13*(3)

Cole, M. A., Kalman, B. A., Pace, T. W., Topczewski, F., Lowrey, M. J., & Spencer, R. L. (2000). Selective blockade of the mineralocorticoid receptor impairs hypothalamic-pituitary-adrenal axis expression of habituation. *Journal of neuroendocrinology, 12*(10), 1034-1042.

Collins, A., Hill, L. E., Chandramohan, Y., Whitcomb, D., Droste, S. K., & Reul, J. M. (2009). Exercise improves cognitive responses to psychological stress through enhancement of epigenetic mechanisms and gene expression in the dentate gyrus. *PLoS ONE, 4*(1), e4330.

Conant, J., Engler, R., Janowsky, D., Maisel, A., Gilpin, E., & LeWinter, M. (1989). Central nervous system side effects of beta-adrenergic blocking agents with high and low lipid solubility. *Journal of cardiovascular pharmacology, 13*(4), 656-661.

Costoli, T., Bartolomucci, A., Graiani, G., Stilli, D., Laviola, G., & Sgoifo, A. (2004). Effects of chronic psychosocial stress on cardiac autonomic responsiveness and myocardial structure in mice. *American journal of physiology.Heart and circulatory physiology, 286*(6), H2133-40.

Dallman, M. F. (2007). Modulation of stress responses: How we cope with excess glucocorticoids. *Experimental neurology, 206*(2), 179-182.

Dallman, M. F., Akana, S. F., Cascio, C. S., Darlington, D. N., Jacobson, L., & Levin, N. (1987). Regulation of ACTH secretion: Variations on a theme of B. *Recent progress in hormone research, 43*, 113-173.

Dallman, M. F., & Jones, M. T. (1973). Corticosteroid feedback control of ACTH secretion: Effect of stress-induced corticosterone ssecretion on subsequent stress responses in the rat. *Endocrinology, 92*(5), 1367-1375.

Dal-Zotto, S., Marti, O., & Armario, A. (2000). Influence of single or repeated experience of rats with forced swimming on behavioural and physiological responses to the stressor. *Behavioural brain research, 114*(1-2), 175-181.

Davis, M. (2006). Neural systems involved in fear and anxiety measured with fear-potentiated startle. *The American Psychologist, 61*(8), 741-756.

De Boer, S. F., Koopmans, S. J., Slangen, J. L., & Van der Gugten, J. (1990). Plasma catecholamine, corticosterone and glucose responses to repeated stress in rats: Effect of interstressor interval length. *Physiology & Behavior, 47*(6), 1117-1124.

De Boer, S. F., Van der Gugten, J., & Slangen, J. L. (1989). Plasma catecholamine and corticosterone responses to predictable and unpredictable noise stress in rats. *Physiology & Behavior, 45*(4), 789-795.

De Goeij DC, , Jezova, D., & Tilders, F. (1992). Repeated stress enhances vasopressin synthesis in corticotropin releasing factor neurons in the paraventricular nucleus. *Brain research, 577*(1)

de Kloet, E. R. (2000). Stress in the brain. *European journal of pharmacology, 405*(1-3), 187-198.

Debiec, J., & Ledoux, J. (2004). Disruption of reconsolidation but not consolidation of auditory fear conditioning by noradrenergic blockade in the amygdala. *Neuroscience, 129*(2)

Deinzer, R., Kirschbaum, C., Gresele, C., & Hellhammer, D. H. (1997). Adrenocortical responses to repeated parachute jumping and subsequent h-CRH challenge in inexperienced healthy subjects. *Physiology & Behavior, 61*(4), 507-511.

Dobrakovova, M., & Jurcovicova, J. (1984). Corticosterone and prolactin responses to repeated handling and transfer of male rats. *Experimental and clinical endocrinology, 83*(1), 21-27.

Dobrakovova, M., Kvetnansky, R., Oprsalova, Z., & Jezova, D. (1993). Specificity of the effect of repeated handling on sympathetic-adrenomedullary and pituitary-adrenocortical activity in rats. *Psychoneuroendocrinology, 18*(3), 163-174.

Dooley, D. J., Bittiger, H., Hauser, K. L., Bischoff, S. F., & Waldmeier, P. C. (1983). Alteration of central alpha 2- and beta-adrenergic receptors in the rat after DSP-4, a selective noradrenergic neurotoxin. *Neuroscience, 9*(4), 889-898.

Duvarci, S., Nader, K., & Ledoux, J. (2005). Activation of extracellular signal-regulated kinase- mitogen-activated protein kinase cascade in the amygdala is required for memory reconsolidation of auditory fear conditioning. *The European journal of neuroscience, 21*(1)

Ehrhart-Bornstein, M., & Bornstein, S. (2008). Cross-talk between adrenal medulla and adrenal cortex in stress. *Annals of the New York Academy of Sciences, 1148*

Elman, M. J., Sugar, J., Fiscella, R., Deutsch, T. A., Noth, J., Nyberg, M., Packo, K., & Anderson, R. J. (1998). The effect of propranolol versus placebo on resident surgical performance. *Transactions of the American Ophthalmological Society, 96*, 283-91; discussion 291-4.

Fanselow, M., & Poulos, A. (2005). The neuroscience of mammalian associative learning. *Annual Review of Psychology, 56*

Ferry, B., & McGaugh, J. (1999). Clenbuterol administration into the basolateral amygdala post-training enhances retention in an inhibitory avoidance task. *Neurobiology of learning and memory, 72*(1)

Ferry, B., Roozendaal, B., & McGaugh, J. L. (1999). Basolateral amygdala noradrenergic influences on memory storage are mediated by an interaction between beta- and alpha1-adrenoceptors. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 19*(12), 5119-5123.

Garcia, A., Marti, O., Valles, A., Dal-Zotto, S., & Armario, A. (2000). Recovery of the hypothalamic-pituitary-adrenal response to stress. effect of stress intensity, stress duration and previous stress exposure. *Neuroendocrinology, 72*(2), 114-125.

Gerra, G., Zaimovic, A., Mascetti, G. G., Gardini, S., Zambelli, U., Timpano, M., Raggi, M. A., & Brambilla, F. (2001). Neuroendocrine responses to experimentally-induced psychological stress in healthy humans. *Psychoneuroendocrinology, 26*(1), 91-107.

Giralt, M., Garcia-Marquez, C., & Armario, A. (1987). Previous chronic ACTH administration does not protect against the effects of acute or chronic stress in male rats. *Physiology & Behavior, 40*(2), 165-170.

Girotti, M., Pace, T. W., Gaylord, R. I., Rubin, B. A., Herman, J. P., & Spencer, R. L. (2006). Habituation to repeated restraint stress is associated with lack of stress-induced c-fos expression in primary sensory processing areas of the rat brain. *Neuroscience, 138*(4), 1067-1081.

Girotti, M., Weinberg, M. S., & Spencer, R. L. (2007). Differential responses of hypothalamus-pituitary-adrenal axis immediate early genes to corticosterone and circadian drive. *Endocrinology, 148*(5), 2542-2552.

Golier, J. A., Schmeidler, J., Legge, J., & Yehuda, R. (2007). Twenty-four hour plasma cortisol and adrenocorticotropic hormone in gulf war veterans: Relationships to posttraumatic stress disorder and health symptoms. *Biological psychiatry, 62*(10), 1175-1178.

Gomez, F., Houshyar, H., & Dallman, M. F. (2002). Marked regulatory shifts in gonadal, adrenal, and metabolic system responses to repeated restraint stress occur within a 3-week period in pubertal male rats. *Endocrinology, 143*(8), 2852-2862.

Govindarajan, A., Rao, B. S., Nair, D., Trinh, M., Mawjee, N., Tonegawa, S., & Chattarji, S. (2006). Transgenic brain-derived neurotrophic factor expression causes both anxiogenic and antidepressant effects. *Proceedings of the National Academy of Sciences of the United States of America, 103*(35), 13208-13213.

Grissom, N., & Bhatnagar, S. (2008). Habituation to repeated stress: Get used to it. *Neurobiology of learning and memory,*

Grissom, N., Iyer, V., Vining, C., & Bhatnagar, S. (2007). The physical context of previous stress exposure modifies hypothalamic-pituitary-adrenal responses to a subsequent homotypic stress. *Hormones and behavior, 51*(1), 95-103.

Grissom, N., Kerr, W., & Bhatnagar, S. (2007). Noradrenergic receptor activity in the basolateral amygdala (BLA) modulates adaptation to repeated stress. *Society for Neuroscience Abstracts, Program No. 198.13*

Grissom, N., Kerr, W., & Bhatnagar, S. (2008). Struggling behavior during restraint is regulated by stress experience. *Behavioural brain research, 191*(2)

Groves, P. M., & Thompson, R. F. (1970). Habituation: A dual-process theory. *Psychological review, 77*(5), 419-450.

Gunnar, M. R., Connors, J., & Isensee, J. (1989). Lack of stability in neonatal adrenocortical reactivity because of rapid habituation of the adrenocortical response. *Developmental psychobiology, 22*(3), 221-233.

Hatfield, T., & McGaugh, J. L. (1999). Norepinephrine infused into the basolateral amygdala posttraining enhances retention in a spatial water maze task. *Neurobiology of learning and memory, 71*(2), 232-239.

Hauger, R. L., Lorang, M., Irwin, M., & Aguilera, G. (1990). CRF receptor regulation and sensitization of ACTH responses to acute ether stress during chronic intermittent immobilization stress. *Brain research, 532*(1-2), 34-40.

Hedou, G., Pryce, C., Di Iorio, L., Heidbreder, C. A., & Feldon, J. (2001). An automated analysis of rat behavior in the forced swim test. *Pharmacology, biochemistry, and behavior, 70*(1), 65-76.

Henke, P. G. (1990). Potentiation of inputs from the posterolateral amygdala to the dentate gyrus and resistance to stress ulcers formation in rats. *Physiology & Behavior, 48*(5), 659-664.

Hennessy, J. W., Levin, R., & Levine, S. (1977). Influence of experiential factors and gonadal hormones on pituitary-adrenal response of the mouse to novelty and electric shock. *Journal of comparative and physiological psychology, 91*(4), 770-777.

Hennessy, M. B., & Levine, S. (1977). Effects of various habituation procedures on pituitary-adrenal responsiveness in the mouse. *Physiology & Behavior, 18*(5), 799-802.

Herman, J. P., & Cullinan, W. E. (1997). Neurocircuitry of stress: Central control of the hypothalamo-pituitary-adrenocortical axis. *Trends in neurosciences, 20*(2), 78-84.

Herman, J. P., Figueiredo, H., Mueller, N. K., Ulrich-Lai, Y., Ostrander, M. M., Choi, D. C., & Cullinan, W. E. (2003). Central mechanisms of stress integration: Hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. *Frontiers in neuroendocrinology, 24*(3), 151-180.

Herman, J., Ostrander, M., Mueller, N., & Figueiredo, H. (2005). Limbic system mechanisms of stress regulation: Hypothalamo-pituitary-adrenocortical axis. *Progress in neuro-psychopharmacology & biological psychiatry, 29*(8)

Himmelsbach, C. K., Gerlach, G. H., & Stanton, E. J. (1935). A method for testing addiction, tolerance, and abstinence in the rat. *Journal of Pharmacology and Experimental Therapeutics, 53*, 179--188.

Huff, N., Frank, M., Wright-Hardesty, K., Sprunger, D., Matus-Amat, P., Higgins, E., & Rudy, J. (2006). Amygdala regulation of immediate-early gene expression in the hippocampus induced by contextual fear conditioning. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 26*(5)

Hunt, P. S., Fanselow, M. S., Richardson, R., Mauk, M. D., Freeman, J. H.,Jr, & Stanton, M. E. (2007). Synapses, circuits, and the ontogeny of learning. *Developmental psychobiology, 49*(7), 649-663.

Ishikawa, A., & Nakamura, S. (2003). Convergence and interaction of hippocampal and amygdalar projections within the prefrontal cortex in the rat. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 23*(31), 9987-9995.

Jaferi, A., & Bhatnagar, S. (2006). Corticosterone can act at the posterior paraventricular thalamus to inhibit hypothalamic-pituitary-adrenal activity in animals that habituate to repeated stress. *Endocrinology, 147*(10), 4917-4930.

Jaferi, A., & Bhatnagar, S. (2007). Corticotropin-releasing hormone receptors in the medial prefrontal cortex regulate hypothalamic-pituitary-adrenal activity and anxiety-related behavior regardless of prior stress experience. *Brain research, 1186*, 212-223.

Jaferi, A., Nowak, N., & Bhatnagar, S. (2003). Negative feedback functions in chronically stressed rats: Role of the posterior paraventricular thalamus. *Physiology & Behavior, 78*(3), 365-373.

Jiang, Y., Langley, B., Lubin, F. D., Renthal, W., Wood, M. A., Yasui, D. H., Kumar, A., Nestler, E. J., Akbarian, S., & Beckel-Mitchener, A. C. (2008). Epigenetics in the nervous system. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 28*(46), 11753-11759.

Johnson, L. L., & Moberg, G. P. (1980). Adrenocortical response to novelty stress in rats with dentate gyrus lesions. *Neuroendocrinology, 30*(3), 187-192.

Jones, S. V., Stanek-Rattiner, L., Davis, M., & Ressler, K. J. (2007). Differential regional expression of brain-derived neurotrophic factor following olfactory fear learning. *Learning & memory (Cold Spring Harbor, N.Y.), 14*(12), 816-820.

Kant, G. J., Bunnell, B. N., Mougey, E. H., Pennington, L. L., & Meyerhoff, J. L. (1983). Effects of repeated stress on pituitary cyclic AMP, and plasma prolactin, corticosterone and growth hormone in male rats. *Pharmacology, biochemistry, and behavior, 18*(6), 967-971.

Keen-Rhinehart, E., Michopoulos, V., Toufexis, D., Martin, E., Nair, H., Ressler, K., Davis, M., Owens, M., Nemeroff, C., & Wilson, M. (2008). Continuous expression of corticotropin-releasing factor in the central nucleus of the amygdala emulates the dysregulation of the stress and reproductive axes. *Molecular psychiatry,*

Keim, K. L., & Sigg, E. B. (1976). Physiological and biochemical concomitants of restraint stress in rats. *Pharmacology, biochemistry, and behavior, 4*(3), 289-297.

Kirschbaum, C., Prussner, J. C., Stone, A. A., Federenko, I., Gaab, J., Lintz, D., Schommer, N., & Hellhammer, D. H. (1995). Persistent high cortisol responses to repeated psychological stress in a subpopulation of healthy men. *Psychosomatic medicine, 57*(5), 468-474.

Kvetnansky, R., Pacak, K., Fukuhara, K., Viskupic, E., Hiremagalur, B., Nankova, B., Goldstein, D. S., Sabban, E. L., & Kopin, I. J. (1995). Sympathoadrenal system in stress. interaction with the hypothalamic-pituitary-adrenocortical system. *Annals of the New York Academy of Sciences, 771*, 131-158.

Lang, P. J., & Davis, M. (2006). Emotion, motivation, and the brain: Reflex foundations in animal and human research. *Progress in brain research, 156*, 3-29.

Lee, J., Everitt, B., & Thomas, K. (2004). Independent cellular processes for hippocampal memory consolidation and reconsolidation. *Science (New York, N.Y.), 304*(5672)

Levenson, J., O'Riordan, K., Brown, K., Trinh, M., Molfese, D., & Sweatt, J. (2004). Regulation of histone acetylation during memory formation in the hippocampus. *The Journal of biological chemistry, 279*(39)

Levine, S., Smotherman, W. P., & Hennessy, J. W. (1977). Pituitary-adrenal hormones and learned taste aversion. *Advances in Biochemical Psychopharmacology, 17*, 163-177.

Lightman, S. L. (2008). The neuroendocrinology of stress: A never ending story. *Journal of neuroendocrinology, 20*(6), 880-884.

Lunga, P., & Herbert, J. (2004). 17Beta-oestradiol modulates glucocorticoid, neural and behavioural adaptations to repeated restraint stress in female rats. *Journal of neuroendocrinology, 16*(9), 776-785.

Ma, S., & Morilak, D. A. (2005). Chronic intermittent cold stress sensitises the hypothalamic-pituitary-adrenal response to a novel acute stress by enhancing noradrenergic influence in the rat paraventricular nucleus. *Journal of neuroendocrinology, 17*(11), 761-769.

Ma, X. M., & Lightman, S. L. (1998). The arginine vasopressin and corticotrophin-releasing hormone gene transcription responses to varied frequencies of repeated stress in rats. *The Journal of physiology, 510 (Pt 2)*

Ma, X. M., Lightman, S. L., & Aguilera, G. (1999). Vasopressin and corticotropin-releasing hormone gene responses to novel stress in rats adapted to repeated restraint. *Endocrinology, 140*(8), 3623-3632.

Ma, X., Levy, A., & Lightman, S. (1997). Emergence of an isolated arginine vasopressin (AVP) response to stress after repeated restraint: A study of both AVP and corticotropin-releasing hormone messenger ribonucleic acid (RNA) and heteronuclear RNA. *Endocrinology, 138*(10)

Maier, S. F., Ryan, S. M., Barksdale, C. M., & Kalin, N. H. (1986). Stressor controllability and the pituitary-adrenal system. *Behavioral neuroscience, 100*(5), 669-674.

Maren, S. (2003). What the amygdala does and doesn't do in aversive learning. *Learning & memory (Cold Spring Harbor, N.Y.), 10*(5)

Maren, S. (2005). Synaptic mechanisms of associative memory in the amygdala. *Neuron, 47*(6), 783-786.

Marin, M. T., Cruz, F. C., & Planeta, C. S. (2007). Chronic restraint or variable stresses differently affect the behavior, corticosterone secretion and body weight in rats. *Physiology & Behavior, 90*(1), 29-35.

Marmigere, F., Givalois, L., Rage, F., Arancibia, S., & Tapia-Arancibia, L. (2003). Rapid induction of BDNF expression in the hippocampus during immobilization stress challenge in adult rats. *Hippocampus, 13*(5), 646-655.

Marti, O., Garcia, A., Valles, A., Harbuz, M. S., & Armario, A. (2001). Evidence that a single exposure to aversive stimuli triggers long-lasting effects in the hypothalamus-pituitary-adrenal axis that consolidate with time. *The European journal of neuroscience, 13*(1), 129-136.

McEwen, B. S. (2008). Central effects of stress hormones in health and disease: Understanding the protective and damaging effects of stress and stress mediators. *European journal of pharmacology, 583*(2-3), 174-185.

McGaugh, J. L. (2004). The amygdala modulates the consolidation of memories of emotionally arousing experiences. *Annual Review of Neuroscience, 27*, 1-28.

McGaugh, J., & Roozendaal, B. (2008). Drug enhancement of memory consolidation: Historical perspective and neurobiological implications. *Psychopharmacology,*

McGhee, L. L., Maani, C. V., Garza, T. H., Desocio, P. A., Gaylord, K. M., & Black, I. H. (2009). The effect of propranolol on posttraumatic stress disorder in burned service members. *Journal of burn care & research : official publication of the American Burn Association, 30*(1), 92-97.

McIntyre, C., Hatfield, T., & McGaugh, J. (2002). Amygdala norepinephrine levels after training predict inhibitory avoidance retention performance in rats. *The European journal of neuroscience, 16*(7)

McIntyre, C., Miyashita, T., Setlow, B., Marjon, K., Steward, O., Guzowski, J., & McGaugh, J. (2005). Memory-influencing intra-basolateral amygdala drug infusions modulate expression of arc protein in the hippocampus. *Proceedings of the National Academy of Sciences of the United States of America, 102*(30)

McIntyre, C., Power, A., Roozendaal, B., & McGaugh, J. (2003). Role of the basolateral amygdala in memory consolidation. *Annals of the New York Academy of Sciences, 985*

McIntyre, D. C., Kent, P., Hayley, S., Merali, Z., & Anisman, H. (1999). Influence of psychogenic and neurogenic stressors on neuroendocrine and central monoamine activity in fast and slow kindling rats. *Brain research, 840*(1-2), 65-74.

McQuade, J. M., Tamashiro, K. L., Wood, G. E., Herman, J. P., McEwen, B. S., Sakai, R. R., Zhang, J., & Xu, M. (2006). Deficient hippocampal c-fos expression results in reduced anxiety and altered response to chronic stress in female mice. *Neuroscience letters, 403*(1-2), 125-130.

Merali, Z., Kent, P., Michaud, D., McIntyre, D., & Anisman, H. (2001). Differential impact of predator or immobilization stressors on central corticotropin-releasing hormone and bombesin-like peptides in fast and slow seizing rat. *Brain research, 906*(1-2), 60-73.

Mercer, B., Grissom, N., & Bhatnagar, S. (2006). Repeated swim enhances hypothalamic-pituitary-adrenal (HPA) responses to subsequent homotypic or heterotypic stressors. *Society for Neuroscience Abstracts,*

Miller, C., Campbell, S., & Sweatt, J. (2008). DNA methylation and histone acetylation work in concert to regulate memory formation and synaptic plasticity. *Neurobiology of learning and memory, 89*(4)

Mineka, S., & Gino, A. (1980). Dissociation between conditioned emotional response and extended avoidance performance. *Learning and Motivation, 11*, 476--502.

Miranda, M. I., Rodri Guez-Garci, A. G., Reyes-Lopez, J. V., Ferry, B., & Ferreira, G. (2008). Differential effects of beta-adrenergic receptor blockade in basolateral amygdala or insular cortex on incidental and associative taste learning. *Neurobiology of learning and memory,*

Mitra, R., Jadhav, S., McEwen, B. S., Vyas, A., & Chattarji, S. (2005). Stress duration modulates the spatiotemporal patterns of spine formation in the basolateral amygdala. *Proceedings of the National Academy of Sciences of the United States of America, 102*(26), 9371-9376.

Mitra, R., Vyas, A., Chatterjee, G., & Chattarji, S. (2005). Chronic-stress induced modulation of different states of anxiety-like behavior in female rats. *Neuroscience letters, 383*(3), 278-283.

Mitsushima, D., Yamada, K., Takase, K., Funabashi, T., & Kimura, F. (2006). Sex differences in the basolateral amygdala: The extracellular levels of serotonin and dopamine, and their responses to restraint stress in rats. *The European journal of neuroscience, 24*(11), 3245-3254.

Moguel-Gonzalez, M., Gomez-Palacio-Schjetnan, A., & Escobar, M. L. (2008). BDNF reverses the CTA memory deficits produced by inhibition of protein synthesis. *Neurobiology of learning and memory, 90*(3), 584-587.

Monfils, M. H., Cowansage, K. K., & LeDoux, J. E. (2007). Brain-derived neurotrophic factor: Linking fear learning to memory consolidation. *Molecular pharmacology, 72*(2), 235-237.

Muir, J. L., & Pfister, H. P. (1987). Time course of the corticosterone and prolactin response following predictable and unpredictable novelty stress in rattus norvegicus. *Physiology & Behavior, 40*(1), 103-107.

Muller, J. F., Mascagni, F., & McDonald, A. J. (2006). Pyramidal cells of the rat basolateral amygdala: Synaptology and innervation by parvalbumin-immunoreactive interneurons. *The Journal of comparative neurology, 494*(4), 635-650.

Muller, J. F., Mascagni, F., & McDonald, A. J. (2009). Dopaminergic innervation of pyramidal cells in the rat basolateral amygdala. *Brain structure & function, 213*(3), 275-288.

Muller, U., Mottweiler, E., & Bublak, P. (2005). Noradrenergic blockade and numeric working memory in humans. *Journal of psychopharmacology (Oxford, England), 19*(1), 21-28.

Murchison, C. F., Zhang, X. Y., Zhang, W. P., Ouyang, M., Lee, A., & Thomas, S. A. (2004). A distinct role for norepinephrine in memory retrieval. *Cell, 117*(1), 131-143.

Natelson, B. H., Ottenweller, J. E., Cook, J. A., Pitman, D., McCarty, R., & Tapp, W. N. (1988). Effect of stressor intensity on habituation of the adrenocortical stress response. *Physiology & Behavior, 43*(1), 41-46.

Nesse, R. M., Bhatnagar, S., & Young, E. (2007). The evolutionary origins and functions of the stress response. In G. Fink (Ed.), *The encyclopedia of stress, second edition* (). New York: Academic Press.

O'Donnell, A., Yang, S. H., & Sharrocks, A. D. (2008). MAP kinase-mediated c-fos regulation relies on a histone acetylation relay switch. *Molecular cell, 29*(6), 780-785.

Ohara, M., Cadnapaphornchai, M. A., Summer, S. N., Falk, S., Yang, J., Togawa, T., & Schrier, R. W. (2002). Effect of mineralocorticoid deficiency on ion and urea transporters and aquaporin water channels in the rat. *Biochemical and biophysical research communications, 299*(2), 285-290.

Oliveira, A. M., Wood, M. A., McDonough, C. B., & Abel, T. (2007). Transgenic mice expressing an inhibitory truncated form of p300 exhibit long-term memory deficits. *Learning & memory (Cold Spring Harbor, N.Y.), 14*(9), 564-572.

Ottersen, O. P. (1982). Connections of the amygdala of the rat. IV: Corticoamygdaloid and intraamygdaloid connections as studied with axonal transport of horseradish peroxidase. *The Journal of comparative neurology, 205*(1), 30-48.

Ou, L. C., & Gean, P. W. (2007). Transcriptional regulation of brain-derived neurotrophic factor in the amygdala during consolidation of fear memory. *Molecular pharmacology, 72*(2), 350-358.

Pacak, K., & Palkovits, M. (2001). Stressor specificity of central neuroendocrine responses: Implications for stress-related disorders. *Endocrine reviews, 22*(4), 502-548.

Pacak, K., Palkovits, M., Kopin, I. J., & Goldstein, D. S. (1995). Stress-induced norepinephrine release in the hypothalamic paraventricular nucleus and pituitary-adrenocortical and sympathoadrenal activity: In vivo microdialysis studies. *Frontiers in neuroendocrinology, 16*(2), 89-150.

Pardon, M. C., Gould, G. G., Garcia, A., Phillips, L., Cook, M. C., Miller, S. A., Mason, P. A., & Morilak, D. A. (2002). Stress reactivity of the brain noradrenergic system in three rat strains differing in their neuroendocrine and behavioral responses to stress: Implications for susceptibility to stress-related neuropsychiatric disorders. *Neuroscience, 115*(1), 229-242.

Pardon, M., Ma, S., & Morilak, D. (2003). Chronic cold stress sensitizes brain noradrenergic reactivity and noradrenergic facilitation of the HPA stress response in wistar kyoto rats. *Brain research, 971*(1)

Pfister, H. P. (1979). The glucocorticosterone response to novelty as a psychological stressor. *Physiology & Behavior, 23*(4), 649-652.

Pinnock, S., & Herbert, J. (2001). Corticosterone differentially modulates expression of corticotropin releasing factor and arginine vasopressin mRNA in the hypothalamic paraventricular nucleus following either acute or repeated restraint stress. *The European journal of neuroscience, 13*(3)

Pitman, D. L., Ottenweller, J. E., & Natelson, B. H. (1990). Effect of stressor intensity on habituation and sensitization of glucocorticoid responses in rats. *Behavioral neuroscience, 104*(1), 28-36.

Pitman, R. K., Sanders, K. M., Zusman, R. M., Healy, A. R., Cheema, F., Lasko, N. B., Cahill, L., & Orr, S. P. (2002). Pilot study of secondary prevention of posttraumatic stress disorder with propranolol. *Biological psychiatry, 51*(2), 189-192.

Pizarro, J. M., Lumley, L. A., Medina, W., Robison, C. L., Chang, W. E., Alagappan, A., Bah, M. J., Dawood, M. Y., Shah, J. D., Mark, B., Kendall, N., Smith, M. A., Saviolakis, G. A., & Meyerhoff, J. L. (2004). Acute social defeat reduces neurotrophin expression in brain cortical and subcortical areas in mice. *Brain research, 1025*(1-2), 10-20.

Quevedo, J., Vianna, M., Roesler, R., Martins, M., de-Paris, F., Medina, J., & Izquierdo, I. (2005). Pretraining but not preexposure to the task apparatus prevents the memory impairment induced by blockade of protein synthesis, PKA or MAP kinase in rats. *Neurochemical research, 30*(1)

Rabinak, C. A., & Maren, S. (2008). Associative structure of fear memory after basolateral amygdala lesions in rats. *Behavioral neuroscience, 122*(6), 1284-1294.

Rainbow, T. C., Parsons, B., & Wolfe, B. B. (1984). Quantitative autoradiography of beta 1- and beta 2-adrenergic receptors in rat brain. *Proceedings of the National Academy of Sciences of the United States of America, 81*(5), 1585-1589.

Rand, J. S., Kinnaird, E., Baglioni, A., Blackshaw, J., & Priest, J. (2002). Acute stress hyperglycemia in cats is associated with struggling and increased concentrations of lactate and norepinephrine. *Journal of veterinary internal medicine / American College of Veterinary Internal Medicine, 16*(2), 123-132.

Rattiner, L. M., Davis, M., & Ressler, K. J. (2005). Brain-derived neurotrophic factor in amygdala-dependent learning. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry, 11*(4), 323-333.

Reist, C., Duffy, J. G., Fujimoto, K., & Cahill, L. (2001). Beta-adrenergic blockade and emotional memory in PTSD. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP), 4*(4), 377-383.

Renthal, W., Maze, I., Krishnan, V., Covington, H., Xiao, G., Kumar, A., Russo, S., Graham, A., Tsankova, N., Kippin, T., Kerstetter, K., Neve, R., Haggarty, S., McKinsey, T., Bassel-Duby, R., Olson, E., & Nestler, E. (2007). Histone deacetylase 5 epigenetically controls behavioral adaptations to chronic emotional stimuli. *Neuron, 56*(3)

Reznikov, L. R., Reagan, L. P., & Fadel, J. R. (2008). Effects of acute and repeated restraint stress on gaba efflux in the rat basolateral and central amygdala. *Brain research,*

Rittenhouse, P. A., Lopez-Rubalcava, C., Stanwood, G. D., & Lucki, I. (2002). Amplified behavioral and endocrine responses to forced swim stress in the wistar-kyoto rat. *Psychoneuroendocrinology, 27*(3), 303-318.

Roberson, E. D., English, J. D., Adams, J. P., Selcher, J. C., Kondratick, C., & Sweatt, J. D. (1999). The mitogen-activated protein kinase cascade couples PKA and PKC to cAMP response element binding protein phosphorylation in area CA1 of hippocampus. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 19*(11), 4337-4348.

Rodriguez-Romaguera, J., Sotres-Bayon, F., Mueller, D., & Quirk, G. J. (2009). Systemic propranolol acts centrally to reduce conditioned fear in rats without impairing extinction. *Biological psychiatry,*

Roozendaal, B., Brunson, K., Holloway, B., McGaugh, J., & Baram, T. (2002). Involvement of stress-released corticotropin-releasing hormone in the basolateral amygdala in regulating memory consolidation. *Proceedings of the National Academy of Sciences of the United States of America, 99*(21)

Roozendaal, B., Griffith, Q., Buranday, J., de Quervain DJ, , & McGaugh, J. (2003). The hippocampus mediates glucocorticoid-induced impairment of spatial memory retrieval: Dependence on the basolateral amygdala. *Proceedings of the National Academy of Sciences of the United States of America, 100*(3)

Roozendaal, B., Hahn, E., Nathan, S., de Quervain DJ, , & McGaugh, J. (2004). Glucocorticoid effects on memory retrieval require concurrent noradrenergic activity in the hippocampus and basolateral amygdala. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 24*(37)

Roozendaal, B., Okuda, S., de Quervain DJ, , & McGaugh, J. (2006). Glucocorticoids interact with emotion-induced noradrenergic activation in influencing different memory functions. *Neuroscience, 138*(3)

Roozendaal, B., Okuda, S., Van der Zee, E. A., & McGaugh, J. L. (2006). Glucocorticoid enhancement of memory requires arousal-induced noradrenergic activation in the

basolateral amygdala. *Proceedings of the National Academy of Sciences of the United States of America, 103*(17), 6741-6746.

Roozendaal, B., Quirarte, G., & McGaugh, J. (2002). Glucocorticoids interact with the basolateral amygdala beta-adrenoceptor--cAMP/cAMP/PKA system in influencing memory consolidation. *The European journal of neuroscience, 15*(3)

Roozendaal, B., Schelling, G., & McGaugh, J. (2008). Corticotropin-releasing factor in the basolateral amygdala enhances memory consolidation via an interaction with the beta-adrenoceptor-cAMP pathway: Dependence on glucocorticoid receptor activation. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 28*(26)

Rossato, J., Bonini, J., Coitinho, A., Vianna, M., Medina, J., Cammarota, M., & Izquierdo, I. (2004). Retrograde amnesia induced by drugs acting on different molecular systems. *Behavioral neuroscience, 118*(3)

Ruys, J. D., Mendoza, S. P., Capitanio, J. P., & Mason, W. A. (2004). Behavioral and physiological adaptation to repeated chair restraint in rhesus macaques. *Physiology & Behavior, 82*(2-3), 205-213.

Sandi, C., Cordero, M. I., Ugolini, A., Varea, E., Caberlotto, L., & Large, C. H. (2008). Chronic stress-induced alterations in amygdala responsiveness and behavior--modulation by trait anxiety and corticotropin-releasing factor systems. *The European journal of neuroscience, 28*(9), 1836-1848.

Sandi, C., & Pinelo-Nava, M. T. (2007). Stress and memory: Behavioral effects and neurobiological mechanisms. *Neural plasticity, 2007*, 78970.

Sapolsky, R. M. (2000). Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. *Archives of General Psychiatry, 57*(10), 925-935.

Sapolsky, R. M. (2002). Chickens, eggs and hippocampal atrophy. *Nature neuroscience, 5*(11), 1111-1113.

Sapolsky, R. M. (2003). Stress and plasticity in the limbic system. *Neurochemical research, 28*(11), 1735-1742.

Sapolsky, R. M., Romero, L. M., & Munck, A. U. (2000). How do glucocorticoids influence stress responses? integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine reviews, 21*(1), 55-89.

Sawchenko, P. E., Brown, E. R., Chan, R. K., Ericsson, A., Li, H. Y., Roland, B. L., & Kovacs, K. J. (1996). The paraventricular nucleus of the hypothalamus and the functional neuroanatomy of visceromotor responses to stress. *Progress in brain research, 107*, 201-222.

Schafe, G. E., Swank, M. W., Rodrigues, S. M., Debiec, J., & Doyere, V. (2008). Phosphorylation of ERK/MAP kinase is required for long-term potentiation in anatomically restricted regions of the lateral amygdala in vivo. *Learning & memory (Cold Spring Harbor, N.Y.), 15*(2), 55-62.

Schafe, G., Atkins, C., Swank, M., Bauer, E., Sweatt, J., & Ledoux, J. (2000). Activation of ERK/MAP kinase in the amygdala is required for memory consolidation of pavlovian fear conditioning. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 20*(21)

Schommer, N. C., Hellhammer, D. H., & Kirschbaum, C. (2003). Dissociation between reactivity of the hypothalamus-pituitary-adrenal axis and the sympathetic-adrenal-medullary system to repeated psychosocial stress. *Psychosomatic medicine, 65*(3), 450-460.

Selcher, J. C., Nekrasova, T., Paylor, R., Landreth, G. E., & Sweatt, J. D. (2001). Mice lacking the ERK1 isoform of MAP kinase are unimpaired in emotional learning. *Learning & memory (Cold Spring Harbor, N.Y.), 8*(1), 11-19.

Shen, C. P., Tsimberg, Y., Salvadore, C., & Meller, E. (2004). Activation of erk and JNK MAPK pathways by acute swim stress in rat brain regions. *BMC neuroscience, 5*, 36.

Shobe, J. (2002). The role of PKA, CaMKII, and PKC in avoidance conditioning: Permissive or instructive? *Neurobiology of learning and memory, 77*(3)

Simeon, D., Knutelska, M., Yehuda, R., Putnam, F., Schmeidler, J., & Smith, L. M. (2007). Hypothalamic-pituitary-adrenal axis function in dissociative disorders, post-traumatic stress disorder, and healthy volunteers. *Biological psychiatry, 61*(8), 966-973.

Simpkiss, J. L., & Devine, D. P. (2003). Responses of the HPA axis after chronic variable stress: Effects of novel and familiar stressors. *Neuro endocrinology letters, 24*(1-2), 97-103.

Smriga, M., & Torii, K. (2003). L-lysine acts like a partial serotonin receptor 4 antagonist and inhibits serotonin-mediated intestinal pathologies and anxiety in rats. *Proceedings of the National Academy of Sciences of the United States of America, 100*(26), 15370-15375.

Spencer, R. L., & McEwen, B. S. (1990). Adaptation of the hypothalamic-pituitary-adrenal axis to chronic ethanol stress. *Neuroendocrinology, 52*(5), 481-489.

Stamp, J., & Herbert, J. (2001). Corticosterone modulates autonomic responses and adaptation of central immediate-early gene expression to repeated restraint stress. *Neuroscience, 107*(3)

Stanton, E. J. (1936). Dihydromorphinone hydrochloride (dilaudid): Its tranquilizing potency, respiratory depressant effects and addiction liability, as tested on the rat. *Journal of Pharmacology and Experimental Therapeutics, 56*, 252--263.

Sullivan, G., Apergis, J., Bush, D., Johnson, L., Hou, M., & Ledoux, J. (2004). Lesions in the bed nucleus of the stria terminalis disrupt corticosterone and freezing responses elicited by a contextual but not by a specific cue-conditioned fear stimulus. *Neuroscience, 128*(1)

Sweatt, J. D. (2001). The neuronal MAP kinase cascade: A biochemical signal integration system subserving synaptic plasticity and memory. *Journal of neurochemistry, 76*(1), 1-10.

Tanaka, M., Kohno, Y., Tsuda, A., Nakagawa, R., Ida, Y., Iimori, K., Hoaki, Y., & Nagasaki, N. (1983). Differential effects of morphine on noradrenaline release in brain regions of stressed and non-stressed rats. *Brain research, 275*(1), 105-115.

Thomas, S. A., & Palmiter, R. D. (1998). Examining adrenergic roles in development, physiology, and behavior through targeted disruption of the mouse dopamine beta-hydroxylase gene. *Advances in Pharmacology (San Diego, Calif.), 42*, 57-60.

Thompson, R. F., & Spencer, W. A. (1966). Habituation: A model phenomenon for the study of neuronal substrates of behavior. *Psychological review, 73*(1), 16-43.

Thomson, F., & Craighead, M. (2007). Innovative approaches for the treatment of depression: Targeting the HPA axis. *Neurochemical research,*

Tronson, N. C., Wiseman, S. L., Olausson, P., & Taylor, J. R. (2006). Bidirectional behavioral plasticity of memory reconsolidation depends on amygdalar protein kinase A. *Nature neuroscience, 9*(2), 167-169.

Tsankova, N., Berton, O., Renthal, W., Kumar, A., Neve, R., & Nestler, E. (2006). Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. *Nature neuroscience, 9*(4)

Tsoory, M. M., Vouimba, R. M., Akirav, I., Kavushansky, A., Avital, A., & Richter-Levin, G. (2008). Amygdala modulation of memory-related processes in the hippocampus: Potential relevance to PTSD. *Progress in brain research, 167*, 35-51.

Ulrich-Lai, Y. M., Arnhold, M. M., & Engeland, W. C. (2006). Adrenal splanchnic innervation contributes to the diurnal rhythm of plasma corticosterone in rats by modulating adrenal sensitivity to ACTH. *American journal of physiology.Regulatory, integrative and comparative physiology, 290*(4), R1128-35.

Ushijima, I., Mizuki, Y., Hara, T., Kudo, R., Watanabe, K., & Yamada, M. (1986). The role of adenosinergic, GABAergic and benzodiazepine systems in hyperemotionality and ulcer formation in stressed rats. *Psychopharmacology, 89*(4), 472-476.

Vahl, T. P., Ulrich-Lai, Y. M., Ostrander, M. M., Dolgas, C. M., Elfers, E. E., Seeley, R. J., D'Alessio, D. A., & Herman, J. P. (2005). Comparative analysis of ACTH and corticosterone sampling methods in rats. *American journal of physiology.Endocrinology and metabolism, 289*(5), E823-8.

Vaiva, G., Ducrocq, F., Jezequel, K., Averland, B., Lestavel, P., Brunet, A., & Marmar, C. R. (2003). Immediate treatment with propranolol decreases posttraumatic stress disorder two months after trauma. *Biological psychiatry, 54*(9), 947-949.

Valentino, R. J., & Van Bockstaele, E. (2008). Convergent regulation of locus coeruleus activity as an adaptive response to stress. *European journal of pharmacology, 583*(2-3), 194-203.

Vining, C., Iyer, V., & Bhatnagar, S. (2007). Intracerebroventricular administration of corticotrophin-releasing hormone receptor antagonists produces different effects on hypothalamic pituitary adrenal responses to novel restraint depending on the stress history of the animal. *Journal of neuroendocrinology, 19*(3), 198-207.

Vogel, W. H., & Jensh, R. (1988). Chronic stress and plasma catecholamine and corticosterone levels in male rats. *Neuroscience letters, 87*(1-2), 183-188.

Vyas, A., Jadhav, S., & Chattarji, S. (2006). Prolonged behavioral stress enhances synaptic connectivity in the basolateral amygdala. *Neuroscience, 143*(2), 387-393.

Vyas, A., Mitra, R., Shankaranarayana Rao, B. S., & Chattarji, S. (2002). Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 22*(15), 6810-6818.

Vyas, A., Pillai, A. G., & Chattarji, S. (2004). Recovery after chronic stress fails to reverse amygdaloid neuronal hypertrophy and enhanced anxiety-like behavior. *Neuroscience, 128*(4), 667-673.

Walker, D. L., & Davis, M. (2008). Role of the extended amygdala in short-duration versus sustained fear: A tribute to dr. lennart heimer. *Brain structure & function, 213*(1-2), 29-42.

Waltereit, R., & Weller, M. (2003). Signaling from cAMP/PKA to MAPK and synaptic plasticity. *Molecular neurobiology, 27*(1), 99-106.

Wang, Z., Zhang, B., Wang, M., & Carr, B. I. (2003). Persistent ERK phosphorylation negatively regulates cAMP response element-binding protein (CREB) activity via

recruitment of CREB-binding protein to pp90RSK. *The Journal of biological chemistry, 278*(13), 11138-11144.

Weinberg, M. S., Girotti, M., & Spencer, R. L. (2007). Restraint-induced fra-2 and c-fos expression in the rat forebrain: Relationship to stress duration. *Neuroscience,*

Weinberg, M., Bhatt, A., Girotti, M., Masini, C., Day, H., Campeau, S., & Spencer, R. (2008). Repeated ferret odor exposure induces different temporal patterns of same-stressor habituation and novel-stressor sensitization in both HPA-axis activity and forebrain c-fos expression in the rat. *Endocrinology,*

Wilensky, A., Schafe, G., & Ledoux, J. (2000). The amygdala modulates memory consolidation of fear-motivated inhibitory avoidance learning but not classical fear conditioning. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 20*(18)

Wu, S. L., Hsu, L. S., Tu, W. T., Wang, W. F., Huang, Y. T., Pawlak, C. R., & Ho, Y. J. (2008). Effects of D-cycloserine on the behavior and ERK activity in the amygdala: Role of individual anxiety levels. *Behavioural brain research, 187*(2), 246-253.

Wust, S., Federenko, I. S., van Rossum, E. F., Koper, J. W., & Hellhammer, D. H. (2005). Habituation of cortisol responses to repeated psychosocial stress-further characterization and impact of genetic factors. *Psychoneuroendocrinology, 30*(2), 199-211.

Yehuda, R., & LeDoux, J. (2007). Response variation following trauma: A translational neuroscience approach to understanding PTSD. *Neuron, 56*(1), 19-32.

Yehuda, R., Teicher, M. H., Trestman, R. L., Levengood, R. A., & Siever, L. J. (1996). Cortisol regulation in posttraumatic stress disorder and major depression: A chronobiological analysis. *Biological psychiatry, 40*(2), 79-88.

Zheng, J., Shen, H., Xiong, Y., Yang, X., & He, J. (2008). The beta1-adrenergic receptor mediates extracellular signal-regulated kinase activation via galphas. *Amino acids,*

Zimmerman, J. M., Rabinak, C. A., McLachlan, I. G., & Maren, S. (2007). The central nucleus of the amygdala is essential for acquiring and expressing conditional fear after overtraining. *Learning & memory (Cold Spring Harbor, N.Y.), 14*(9), 634-644.

Zou, Y., Komuro, I., Yamazaki, T., Kudoh, S., Uozumi, H., Kadowaki, T., & Yazaki, Y. (1999). Both gs and gi proteins are critically involved in isoproterenol-induced cardiomyocyte hypertrophy. *The Journal of biological chemistry, 274*(14), 9760-9770.