

2010

Structural and Molecular Correlates of Individual Differences in Anxiety Behavior and the Stress Response

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STRUCTURAL AND MOLECULAR CORRELATES OF INDIVIDUAL
DIFFERENCES IN ANXIETY BEHAVIOR AND THE STRESS RESPONSE

A Thesis Presented to the Faculty of
The Rockefeller University
in Partial Fulfillment of the Requirements for
the degree of Doctor of Philosophy

by

Melinda Miller

June 2010

STRUCTURAL AND MOLECULAR CORRELATES OF INDIVIDUAL DIFFERENCES IN ANXIETY BEHAVIOR AND THE STRESS RESPONSE

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The Rockefeller University 2010

Although many people are exposed to stressful experiences during their lifetime, only 5-35% will be diagnosed with Post-Traumatic Stress Disorder (PTSD). While most animal models of stress are effective at producing behavioral changes in an entire group of animals, they fail to account for individual differences in the human stress response or for the variability in baseline anxiety.

Animal models of acute and chronic stress increase anxiety behavior and induce structural and neurochemical changes in brain regions necessary for learning, memory, fear responses, and executive function; the hippocampus, amygdala and medial prefrontal cortex (mPFC). In the following experiments, we measured basal anxiety levels and compared anxiety behavior before and after stress. Individuals were grouped based on basal anxiety profiles (either “Calm” or “Anxious”) or by whether they developed changes in behavior after stress (stress vulnerable, or “PTSD-like” and stress resilient, or “Resilient”).

Results showed that molecular and morphological differences were already apparent prior to stress. Anxious Sprague Dawley and Lewis rats had shorter apical dendrites in pyramidal neurons of mPFC and anxiety correlated negatively with dendritic arbor size. Densitometry analysis found individual

differences in mRNA expression of the neuropeptide Cocaine-Amphetamine–Regulated-Transcript (CART). CART expression was higher in the hippocampus and medial amygdala of “Calm” individuals, and expression in the hippocampus, central amygdala, mPFC, and orbitofrontal cortex correlated with anxiety behavior. CART expression also differed in individuals in the PTSD-like and Resilient profiles. CART mRNA and protein levels were higher in the hippocampus and lower in several nuclei of the amygdala in stress-resilient animals; while CART levels in the PTSD-like group was lower in the Infralimbic mPFC.

These results emphasize the importance of individual differences in behavior, and show that there are significant structural and molecular differences between adult male rats. They also illustrate the need for experiments that produce both stress-vulnerable and resilient individuals, as resilience may be an active process involving changes to many brain regions. Finally, these results present a novel role for the neuropeptide CART in establishing or predicting individual differences in anxiety, and suggest that CART may be involved in both fear behavior and neuroprotection.

For my parents who always encouraged me to ask “why?”
And for all of the animals, big and small, fluffy and slimy,
that make scientific research possible

Acknowledgements:

I would like to thank Bruce McEwen for sparing his valuable free time to chat about The Rockefeller University graduate program and the finer points of neuroendocrinology with an unknown undergrad working in a cognitive psychology lab who hadn't even applied to graduate school. His passion for science and his desire to educate struck me immediately and convinced me that not only could I jump from the MRI suite to the benchtop, but that he was the type of advisor that I was looking for. As a mentor, Bruce has shown compassion during failures and contagious enthusiasm when mine was temporarily lost. Always available and full of new ideas, he has helped me to remember what doing research is all about, the love of science and the desire to help people.

I thank the entire McEwen Lab, members past and present, all of whom have lent a hand and given me advice over the years. Particularly, I would like to thank Gwendolyn Wood for sharing with me her vast knowledge of the intricacies of rodent behavior, and for helping me with experimental design and troubleshooting. I would also like to thank Richard Hunter for providing me with an education on a little neuropeptide that I would have otherwise overlooked, and for all of his help with techniques and theory. I thank Keith Akama and Elizabeth Waters for their intellectual input, editing assistance, good advice (scientific and otherwise), and moral support over the years. They have been mentors to me in so many ways. I would also like to thank Karen Bulloch for her advice and support, and for providing me with a successful side project that has opened my eyes to the importance of understanding what goes on "below the neck". The

laboratory wouldn't be the same place without Halina Korsun and Adelaide Acquaviva. I have greatly benefitted from their open-door policy; whether I needed administrative help, someone to listen to me rant, a good laugh or a small piece of chocolate, they have always been there for me.

I have been fortunate enough to have the assistance of many summer students and technicians, without whom I could have not completed my research. I thank Stephanie da Silva, Brian Harfenist, Riley Guerin, Eva Hudgins, Katherine McCarthy, Sarah Filipski and Athena Wang for their willingness to cut brains for weeks, analyze data in the dark, clean countless dirty rat cages, and for speaking softly and caring tenderly for the laboratory animals. I would especially like to thank Athena Wang for taking me under her wing in my first year of school and teaching me everything that I know about doing benchwork in a biology lab.

My faculty advisory committee has been an invaluable resource in forming and reforming my thesis project. I thank Mary Jeanne Kreek and Donald Pfaff for their helpful advice and for being proponents and comrades in the fight for animal facility reform. I thank Regina Sullivan for serving as my external committee member, and for taking the time to review my thesis and participate in my defense examination. I have gratitude and sincere appreciation for Sid Strickland and the entire Dean's office for their helpful advice, accessibility when answering my questions, and for all of the free snacks.

I would also like to acknowledge Joseph LeDoux for establishing the Conte Center for the Neuroscience of Fear and Anxiety (supported by the National Institutes of Health 5P50MH58911), which enabled me to meet people

from many fields who share the same interests in the biology of stress; from clinical to molecular biology. I also would like to acknowledge the support of NIH MH41256 and Lundbeck Research, USA. As part of these collaborations, I thank John Morrison, Patrick Hof and the entire Morrison and Hof laboratories for providing technical support and sharing their equipment and facility with me so easily. In particular, I owe much gratitude to Daniel Christoffel, Bill Janssen, and Jason Radley, and Dara Dickstein for teaching me techniques for cell-loading and perfusions, and for making me feel like a member of the family while I spent time in their lab.

Finally, I would like to thank my dear friends and family for their support over the years. I thank my husband, Lee, who often sleeps alone at night while I work odd hours in the lab and always has a shoulder ready for me to lean on when I need it. I thank my sister, Beth, for sharing in my love of science and for always enjoying a rat dissection story over the dinner table. I also thank my parents for their continued cheerleading and (sometimes not so) gentle chastising at my procrastination and for fostering my love of learning. When I think of my childhood, I can only conclude that there was as much “nurture” as “nature” involved in making me the fittest I could be, and I feel very fortunate to be surrounded with so much love and encouragement.

Table of Contents

Chapter 1: Introduction	1
What is stress?	1
Post-Traumatic Stress Disorder	2
Fear in the Brain - The amygdala.....	4
Amygdala Modulation of the Hippocampus.....	5
Bidirectional modulation of the amygdala and the prefrontal cortex.....	7
Acute and Chronic Stress Models.....	9
Models of predator stress	12
Individual differences in anxiety and the stress response	13
Modulators of the stress response	18
Glucocorticoids.....	18
Catecholamines.....	20
Serotonin.....	22
Glutamate.....	23
The role of small neuropeptides.....	24
NPY and its receptors	24
CART Neuropeptide	26
Current Aims	29
 Chapter 2: Materials and Methods	 32
Animals	32
Stress.....	32
Restraint Stress.....	32
Immobilization Stress.	33
Cat Odor Exposure Stress.....	33
Multimodal Stress.....	34
Blood collection.....	35
Tail blood collection.....	35
Trunk blood collection.	35
Corticosterone Assay.	35
Behavioral testing.	36
Open Field.....	36
Elevated plus maze.	36
Cat odor exposure behavior.	36
Home cage behavior and social interaction:.....	37
Sucrose preference:	37
Decapitation:	37
Perfusion:.....	37
Cell-loading:	38
Cell tracing and analysis:	38
Immunohistochemistry:	39
In situ hybridization:	41
Densitometry analysis:	43
Corticosterone modulation of stress resilience:.....	44

NMDA-receptor modulation of stress resilience	44
Neuropeptide Y, Y5 receptor experiment:	45
Chapter 3: Small neuropeptides present different profiles based on stressor timing and modality.....	46
Experimental Paradigm	47
Results	48
Behavior	48
CART neuropeptide.....	48
Neuropeptide Y (NPY).....	54
Neuropeptide Y receptor Y5	58
Discussion.....	61
Chapter 4: The structural and molecular correlates of individual differences in control anxiety profiles	66
Experimental Paradigm	67
Experiment 1: Analysis of dendritic arborization of pyramidal neurons in layers II/III of the medial prefrontal cortex after a single acute restraint stress and in control animals with behavioral extremes.	68
Experiment 2: Analysis of mRNA expression of small peptides in control animals with behavioral extremes.	69
Results	70
Experiment 1	70
Experiment 2	85
Discussion.....	93
Chapter 5: Individual differences in an animal model of Post-Traumatic Stress Disorder	98
Experimental Paradigm	99
Using the multimodal stress episode with a conditioned cue as a model of PTSD.....	99
Separation of affected individuals by examining changes to anxiety behavior over time.....	100
Molecular analysis.....	101
Results	102
The multimodal stressor: Lewis vs. Fischer rats.....	102
Using the multimodal stress episode with a conditioned cue as a model of PTSD in Lewis rats.....	104
Separating out the affected: PTSD-like vs. Resilient behavior	108
CART Neuropeptide molecular profile in PTSD-like and Resilient animals	112
Discussion	127

Chapter 6: Mechanisms for stress resilience and prevention of PTSD	135
Experimental Paradigm.....	136
Experiment 1: NMDA-Receptor blockade during an acute multimodal stress exposure.....	136
Experiment 2: Noninvasive corticosterone treatment during an acute multimodal stress exposure.....	136
Experiment 3: Y5 receptor antagonist treatment during chronic stress:	137
Results	138
Experiment 1	138
Experiment 2	141
Experiment 3	144
Discussion	157
 Chapter 7: General discussion and implications for stress research	 163
The brain encodes for different aspects of a stressor, including length, timing and modality.....	164
Individual differences in basal levels of anxiety correlate with morphological differences in rat mPFC.	165
What causes individual differences in anxiety?	169
Stress resilience is an active process	172
The benefits and pitfalls of the multimodal stress paradigm.....	173
The role of CART in fear and anxiety behavior	175
Location and connectivity of CART to other neurotransmitter and hormone systems	176
CART has multiple functions in the brain.....	181
Future directions and conclusions.....	187
 Appendix 1: Regions of interest used for densitometry	 191
 References	 196

List of Figures

Figure 1. Post-stress elevated plus maze behavior.	50
Figure 2. CART mRNA expression changes with stressor timing and modality	52
Figure 3. NPY mRNA expression changes with stressor timing and modality	56
Figure 4. Y5 mRNA expression does not change with stress.	59
Figure 5. Factors for separating groups based on anxiety behavior.	72
Figure 6. Individual behavior in the elevated plus maze correlated with mPFC dendritic length regardless of stress.	74
Figure 7. Confirmation of selection criteria for Calm and Anxious groups for both Lewis and Sprague-Dawley (SD) rat strains.	79
Figure 8. The size of the apical dendritic arbors differ between Calm and Anxious extremes in the prelimbic area of the mPFC in two strains of rats.	80
Figure 9. Sholl Analysis reveals overall differences in the distribution of dendritic material along the apical dendrite of pyramidal neurons of the prelimbic area of the mPFC.	81
Figure 10. Other factors that contribute to the anxiety behavior and dendritic length differences found in both strains of rats	83
Figure 11. Control groups divided by anxiety behavior differ in CART mRNA expression in the hippocampus and amygdala.	88
Figure 12. Control groups divided by anxiety behavior differ in CART mRNA expression in the prefrontal cortex.	90
Figure 13. CART mRNA expression correlates with individual anxiety behavior in the elevated plus maze.	91
Figure 14. The multimodal stress episode is sufficient for producing a corticosterone response and behavioral changes seven days later	103
Figure 15. Playing a stress-associated audio cue during behavioral testing in the elevated plus maze does not further enhance the long-term effects of a multimodal stressor	106

Figure 16. Factors for separating groups based on anxiety behavior	110
Figure 17. Higher CART mRNA expression levels were found in the hippocampus and central amygdala in stress-resilient individuals.....	114
Figure 18. Lower CART mRNA expression levels were found in the prelimbic region of the mPFC in stress-resilient individuals.....	115
Figure 19. CART peptide expression in the hippocampal formation, amygdala and prefrontal cortex.....	120
Figure 20. Higher CART protein expression levels were found in the hippocampus in stress-resilient individuals.....	122
Figure 21. Lower CART protein expression levels were found in the nuclei of the amygdala in stress-resilient individuals	124
Figure 22. Lower CART protein expression levels were found in the infralimbic region of mPFC in PTSD-like individuals.....	126
Figure 23. Effects of NMDA receptor antagonist on acute multimodal stress remain unclear.....	140
Figure 24. Post-stress plasma Corticosterone (CORT) is inhibited by 12 hours of CORT treatment	142
Figure 25. Effects of CORT treatment on the acute multimodal stress appear unclear.....	143
Figure 26. Chronic restraint stress reduces weight gain regardless of Y5 receptor antagonist treatment.....	147
Figure 27. Restraint stress and Y5 receptor antagonist treatment have no effect on sucrose preference or in cage aggression.....	148
Figure 28. Chronic stress and/or chronic Y5 antagonist treatment produced little change in anxiety behavior	149
Figure 29. Chronic Restraint Stress reduces NPY mRNA expression in the hippocampus and Y5 antagonist treatment appears to partially reverse these effects.....	153
Figure 30. Both chronic restraint stress and Y5 antagonist appear to influence Y5 receptor mRNA expression.....	155

Supplementary Figure 1. Effects of husbandry on weight gain, anxiety and dendritic morphology in the mPFC.....	189
Supplementary Figure 2. Autoradiograph examples of regions sampled by densitometry for mRNA expression.....	194

List of Tables

Table 1. A summary of known anxiety related and stress-induced changes in mPFC.....	168
Table 2. A summary of CART mRNA and protein expression findings in the brain.....	180
Table 3. The functional implications of CART peptide expression in regions with consistent expression profiles.....	185
Table 4. Possible implications of CART expression in regions with conflicting expression.....	186

List of Abbreviations

5-HT: serotonin
ACTH: adrenocorticotrophic hormone
AIS: acute immobilization stress
ANOVA: analysis of variance
BLA: basolateral nucleus of the amygdala
BNST: bed nucleus stria terminalis
BSTIA: bed nucleus stria terminalis Intraamygdaloid division
CA: cornu Ammonis
CART: Cocaine-Amphetamine-Regulated-Transcript
CE: central nucleus of the amygdala
CIS: chronic immobilization stress
CORT: corticosterone
COS: cat odor stress
CRH: corticotropin-releasing hormone
CRS: chronic restraint stress
DAB: 3,3'-diaminobenzidine
dATP: 2'-Deoxyadenosine 5'-triphosphate
EPM: elevated plus maze
fMRI: functional magnetic resonance imaging
GABA: gamma-Aminobutyric acid
GCL: granule cell layer
HIL: hilus
HPA: hypothalamic-pituitary-adrenal axis
IL: infralimbic region
i.p.: intraperitoneal
LA: lateral nucleus of the amygdala
LC: locus coeruleus
ME: medial nucleus of the amygdala
mPFC: medial prefrontal cortex
mRNA: messenger ribonucleic acid
NMDA: N-methyl-D-aspartate
NPY: Neuropeptide Y
OFC: orbitofrontal cortex
PAG: periaqueductal gray
PL: prelimbic region
PTSD: Post-Traumatic Stress Disorder
RIA: radioimmunoassay
RNA: ribonucleic acid
s.c.: subcutaneous
SD: Sprague-Dawley
SGZ: subgranular zone
siRNA: small interfering ribonucleic acid
SSRI: selective serotonin reuptake inhibitor

Chapter 1: Introduction

What is stress?

Practically everyone has an understanding of what stress is, at least in terms of the modern world; however, this term can be somewhat hard to define. Stress can be thought of as the organism's response to threatening stimuli in the environment, either real or perceived, that increases production of hormones such as cortisol and adrenaline, and activates many other neurochemical systems in the brain. These produce a cascade of changes that initiate the "fight or flight" response, essentially the immediate upregulation of the sympathetic nervous system and the temporary inhibition of parasympathetic function (de Kloet et al. 2005). In the short term, this helps the organism to adapt, accommodate to, or escape from the stressor and bring the body back into balance. This process is also called "allostasis", meaning establishing stability through change (McEwen 2004). The release of the stress hormones CRH, ACTH, and cortisol, as well as the neurotransmitters epinephrine, norepinephrine, and serotonin during a stressful event can help the body to produce enough energy to quickly escape from aversive stimuli (Sandi et al. 1992) or predators (Adamec et al. 1998). Acutely, these neurochemicals have also been shown to boost the immune system, increase attention and even enhance memory (McEwen 2004). However, during chronic stress these same systems work overtime, which causes wear and tear on the body and results in increased "allostatic load" or "overload" (McEwen et al. 1993). In contrast with acute stress, chronic stress can reduce energy, disrupt mood, weaken

the immune system, and impair attention and memory processes (McEwen 2003, 2006).

Post-Traumatic Stress Disorder

Occasionally, a single stressor can be so severe and traumatic, or the body can adapt so poorly, that it can create long-term imbalance in the systems involved in allostasis. Post Traumatic Stress Disorder (PTSD) is a condition contingent upon a physical or psychosocial stressor that is remembered or re-experienced with such emotional strength, that people continue to have intensely fearful, anxious, and dysphoric responses well after the threat is gone (Bonne et al. 2004). In the United States, there is a 6.8% lifetime prevalence for developing PTSD (Kessler et al. 2005). Yehuda and LeDoux (2007) have classified the common symptoms of PTSD into three types: 1. reexperiencing symptoms; the uncontrollable intrusions of memories for the traumatic event that coincide with the same physiological responses associated with the original trauma, such as flashbacks and nightmares; 2. avoidance symptoms, intentional avoidance of reminders of the traumatic event, and may include general avoidance of social and emotional situations; and 3. hyperarousal symptoms, physiological responses on par with stress-state arousal when no stressors are present. These include hypervigilance, increased startle response and a general state of fearfulness. Treatment options for PTSD symptoms include exposure therapy, psychotherapy, as well drugs to treat other forms of anxiety and typical and atypical antidepressants; however, most treatments have less than ideal response rate (Seidler et al. 2006; Hofmann et al. 2008; Donovan et al. 2009).

Animal models have commonly been used to elucidate the biological mechanisms of neurological diseases and disorders. Using animals to study PTSD is particularly challenging because many of the psychological symptoms that characterize the disorder, such as flashbacks, nightmares, and anxiety towards complex stimuli, are difficult to reproduce outside of the human species. Nonetheless, the major structures thought to be involved in processing fearful stimuli, producing a stress response, and encoding emotional memory are shared across mammalian species.

The major structures involved in the stress response - the amygdala, hippocampus and prefrontal cortex - have an intricate connectivity that controls and modulates the behavioral stress response. These generate a series of checks and balances via excitatory connections and inhibitory feedback loops. Likewise, anxiety behaviors have been identified that are shared across species, including hyper-vigilance to novel stimuli (Mikics et al. 2008; Grillon et al. 2009), and enhanced acoustical startle reflex (Garrick et al. 2001; Cohen et al. 2004), and social withdrawal (Morilak et al. 2005; Adamec et al. 2007). Although non-invasive neuroimaging techniques have begun to develop the resolution to examine connectivity between areas involved in the processing of emotionally arousing events, animal research is still the cornerstone to understanding the anatomical, functional, and molecular pathways through which these brain structures communicate.

Fear in the Brain - The amygdala

Several decades of research has helped to validate the role of the amygdala as the locus of fear processing and fear learning in the brain (Fanselow et al. 1999). Bilateral lesions or pharmacological inactivation of the amygdala before training eliminate the fear conditioning response (LeDoux et al. 1988; Muller et al. 1997), and temporary inactivation after fear conditioning can prevent consolidation of a fear memory, impairing subsequent memory recall (Sacchetti et al. 1999). Further, bilateral amygdala lesions many days after fear conditioning can completely abolish the memory, demonstrating that the amygdala is also a site of fear memory storage (Maren et al. 1996). Although less pronounced in humans, bilateral loss of the amygdala limits the production of an appropriate autonomic fear response during conditioning, and also impairs the comprehension of social cues, such as the recognition of fearful and angry facial expressions (Adolphs et al. 1994; LaBar et al. 1995; Adolphs et al. 1998).

The amygdala is divided into several nuclei that are involved in input of sensory information, fear processing and coincidence detection, and output of fear behavior (Pitkanen et al. 1997). The lateral nucleus of the amygdala (LA) is the region that receives the heaviest sensory input, mainly from thalamic nuclei. LA projects to almost all other subnuclear regions within the amygdala. It is thought that most of the processing and consolidation of fear learning occurs here, as lesions specific to LA, or the slightly larger lateral/basolateral amygdala (LA/BLA) complex, prevent the consolidation of fear conditioning (Nader et al. 2001). Projections from LA form separate fiber tracts that innervate the Basal, Accessory Basal, Medial

(ME) and Central (CE) subnuclei of the amygdala. Processing of information received from LA occurs in parallel within each of the other subnuclei, and then converges into the major output region, CE. Neurons in CE project to the brainstem, hypothalamus and higher cortical areas, signaling the production of stress hormones and a behavioral response (Bohus et al. 1996; Salome et al. 2001). CE is required for the elicitation of a learned fear response, and CE specific lesions prevent animals from producing conditioned behavior (Nader et al. 2001).

LA and the other subnuclei also receive projections from hippocampal and parahippocampal structures, the thalamus, and discrete regions of the prefrontal cortex that can modulate amygdalar activity (Ino et al. 1990; Szinyei et al. 2000; Furtak et al. 2007). Additionally, there are interneurons that send feedback to and from the subnuclei as during stimulus processing (Washburn et al. 1992; Royer et al. 2002). Together these connections form a complex network responsible for producing appropriate and highly specific behavioral responses to changes in the environment.

Amygdala Modulation of the Hippocampus

While hippocampal projections to the amygdala may modulate early stimulus processing (Maren et al. 1995), the influence of amygdalar activity on hippocampal function and hippocampal-dependent memory is more extensive. The ability to induce long term potentiation (LTP) in the brain has been utilized as a measure of neuroplasticity and learning. Conditioning a rat to respond to a neutral stimulus, such as a tone, when it is paired with an innately aversive footshock, induces LTP in LA, which coincides with the learning of the new fear contingencies (Rogan et al.

1997). An increase in amygdala activity can increase activity in the hippocampus, specifically enhancing LTP in the dentate gyrus, and lesions in the amygdala impair LTP induction (Ikegaya et al. 1995; McGaugh 2002).

Behaviorally, amygdala modulation of the hippocampus is even more evident. In animals, amygdala activity has been demonstrated to both enhance and impair memory for spatial tasks, inhibitory avoidance, context conditioning, appetitive conditioning and reward learning (Izquierdo et al. 1997; McGaugh 2002; Hayes et al. 2004; Richter-Levin 2004). Studies employing pharmacological excitation of the amygdala using amphetamines and glucocorticoids injected before and after training have shown increases in accuracy in the Morris water maze and inhibitory avoidance tasks (Packard et al. 1998; Roozendaal 2000). The pairing of acute stressors shown to temporarily increase amygdala activity, such as footshock and restraint stress with the Morris water maze, have demonstrated both positive and negative effects on performance (Luine et al. 1996; de Quervain et al. 1998; Kim et al. 2001; Shors 2001). Lesions to the amygdala or temporary inhibition of amygdala activity prevent these stress-induced alterations in memory performance and often cause impairment (Kim et al. 2001; Kim et al. 2005), demonstrating that stress is a complex stimuli that activates much more than the amygdala.

In humans, amygdala activity also has been confirmed to enhance hippocampal dependent memory. Memory for arousing words and events is generally better than for emotionally neutral ones (Phelps et al. 1997), and pairing neutral words or stories with emotionally arousing ones enhances recollection for the neutral stimuli (Maratos et al. 2001). Although people with amygdala damage

can remember arousing and non-arousing stimuli as well as control subjects, they do not show the recall enhancement for the emotionally arousing stimuli (Anderson et al. 2001). Functional Magnetic Resonance Imaging (fMRI) evidence suggests that an image is more likely to be remembered if there is greater amygdala activation, and if amygdala activity correlates with hippocampal activity during the original encoding (Dolcos et al. 2004). However, people with PTSD have exaggerated amygdala activation for trauma-associated stimuli that does not habituate even after many presentations (Protopopescu et al. 2005). While further research is needed, it is possible that amygdala-hippocampal interactions during encoding and consolidation of a traumatic event may be a major factor in the vivid recollection and enduring properties of PTSD related memories.

Bidirectional modulation of the amygdala and the prefrontal cortex

Major projections from the prefrontal cortex (PFC) innervate the amygdala. These connections are largely inhibitory, or GABAergic, with the bulk of the projections converging on inhibitory interneurons in the LA/BLA and CE (McDougall et al. 2004; Vertes 2004). Fiber tracts originate from several areas, including the anterior cingulate (ACg), prelimbic (PL) and infralimbic (IL) region. By inhibiting activity in LA/BLA, these projections inhibit aversive conditioning and the elicitation of fear responses in the presence of previously conditioned stimuli. This effect is called extinction, and it is a gradually learned process thought to be governed by the more medial areas of PFC (mPFC) in rodents (Maren et al. 2004). Lesions in mPFC and ACg prevent extinction learning (Morgan et al. 1993) and

electrophysiological studies show that LTP in mPFC is necessary to maintain extinction (Herry et al. 2002).

Extinction is a two-way street. When a neutral stimulus does not continue to predict an aversive outcome, the memory trace detecting this “coincidence” in the amygdala ceases to be reinforced with electrophysiological and molecular activity. As a result, mPFC, which receives inhibitory input from many areas including the hippocampus and amygdala, becomes more active as these regions become less active (Quirk et al. 2003). This allows for stronger inhibitory input from mPFC to the amygdala.

This feedback loop has been confirmed in human extinction learning. Using fMRI, a similar negative correlation has been found between amygdala and prefrontal activation during extinction learning in healthy subjects (Phelps et al. 2004). This suggests that the neural correlates of fear learning and extinction are preserved across species. It has been suggested that people with anxiety disorders may have an enhanced amygdala response to threatening stimuli due to poor inhibitory tone from a less active PFC. This may lead to reduced inhibition of the amygdala during memory encoding, which could give heightened emotional significance to neutral environmental stimuli, and cause symptoms of anxiety disorders (Protopopescu et al. 2005). This would also impair future memory extinction, possibly preventing these fear-associated memories from being properly extinguished once the threat is gone (Quirk et al. 2003). Irregularities in both encoding and extinction may be possible in PTSD.

Acute and Chronic Stress Models

The amygdala, hippocampus and PFC, supplemented by other connected brain structures are responsible for creating fear-related adaptive responses to the environment. By examining simple models of animal stress, we can better understand how physical and behavioral symptoms develop after a traumatic episode and determine ways to reverse a maladaptive stress response. Acute and chronic stressors have been extensively studied in the rodent. The most common stress methods are the use of restraint or immobilization, social interaction and defeat, and predator or predator odor presentation. In addition, electrical shocks to the feet or tail are employed in acute stress studies, and even fear conditioning can be thought of as an acute stressor.

The hippocampus is one of the few areas of known adult neurogenesis. Newly proliferating neurons in the dentate gyrus seem particularly vulnerable to the effects of stress, making the hippocampus one of the most variable regions in the brain. Chronic restraint stress has been shown to inhibit neurogenesis in the granule cell layer and decrease cell survival by almost 50 percent after six weeks of stress (Pham et al. 2003). In the surviving neurons and neurons from surrounding regions, chronic stress results in considerable dendritic remodeling. Repeated restraint, social subordination, and predator exposure all produce apical dendritic remodeling in hippocampal CA3, resulting in fewer branch points, shorter mean dendritic length and reduced spine density (Magarinos et al. 1996; McEwen 1999; Diamond et al. 2000; Vyas et al. 2002). The hippocampus has a major role in the consolidation of memory (Morris et al. 1990; Izquierdo et al. 2000; Holscher 2003).

As would be predicted by the structural and electrophysiological data, chronic stress produces deficits in spatial memory and impairments in other hippocampal-dependent learning paradigms, including inhibitory avoidance and context conditioning (Izquierdo et al. 1997; de Quervain et al. 1998; McEwen 1999). Fortunately, once the stress has ceased, most of these effects are reversible over time (McEwen 1999), which gives hope to reversing the effects caused by long-term stress related disorders in humans.

In the PFC, chronic stress results in similar dendritic remodeling. Repeated restraint stress produces a reduction in apical dendritic length and loss of spine density on pyramidal neurons in ACg, PL and IL regions of the mPFC (Radley et al. 2004; Liston et al. 2006; Radley et al. 2006; Goldwater et al. 2009) . However, the chronic stress induced dendritic atrophy is reversible if the animals are given several weeks to recover (Radley et al. 2005); but, there is some evidence that the original dendritic morphology is permanently altered (Goldwater et al. 2009). In a test of working memory, chronic stress induced memory impairments, and poor task performance correlated with shorter apical dendrites in mPFC (Liston et al. 2006).

In contrast, dendritic hypertrophy is found in the amygdala following chronic stress. A 10-day immobilization stress paradigm produced an increase in dendritic length and total number of branch points on apical dendrites of BLA pyramidal-like neurons (Vyas et al. 2002), as well as increased spine density along the apical dendrite (Mitra et al. 2005). An increase in anxiety behavior was also found in the same animals. A significant aspect of the dendritic hypertrophy in BLA is that it does not appear to be readily reversible once the stress has ceased, at least not

after the same amount of recovery time sufficient to reverse CA3 and mPFC stress-induced atrophy (Vyas et al. 2004). The potential failure of amygdala neurons to return to a pre-stress state may be a key reason why the symptoms of PTSD and other stress disorders often diminish very slowly and sometimes not at all

Although the effects are smaller and more discrete, a single acute stress also appears to differentially affect dendritic morphology in several brain regions. Acute stress-induced changes in the hippocampus have been previously examined in both male and female rats (Shors et al. 2001). In males, apical dendritic spine density in CA1 pyramidal neurons increased 24 hours after acute tailshock, whereas spine density decreased in the apical dendrites of diestrus females. Regardless of the sex differences, this suggests that the hippocampus can rapidly adapt to stress through structural modifications. Long-enduring structural changes after acute stress have also been demonstrated in the hippocampus. Three weeks after a brief social defeat stress, a reduction in apical dendritic length of CA3 pyramidal neurons was still evident (Kole et al. 2004).

In the amygdala, a single two-hour immobilization stress was shown to increase spine density on primary branches of the apical dendrite 10 days after stress, but not just 24 hours post-stress (Mitra et al. 2005). This correlated with a delayed enhancement in anxiety behavior in the same animals. There are no published studies examining the effect of acute stressors on mPFC dendritic morphology, although a 10-minute restraint stress repeated for one week was sufficient to reduce the length of proximal branches on the apical dendrites of pyramidal neurons (Brown et al. 2005).

Models of predator stress

In a more naturalistic model of stress exposure, rodents have been subjected to their natural predators, namely cats, foxes and ferrets, and examined for long term behavioral and neurochemical changes. Adamec and Shallow (1993) reported a long-lasting increase in anxiety-like behavior and a decrease in risk assessment in the elevated plus maze (EPM) following a single 5-minute direct exposure of a rat to a cat. These effects could be seen 30 minutes to 1 hour after predator exposure and persisted for at least 3 weeks in hooded rats. Likewise, acute predator stress increased acoustic startle response and decreased entries into the light box of the light-dark box paradigm (Blundell et al. 2005) and potentiated neural transmission both to and from the amygdala 10 days post-stress (Adamec et al. 2005). Chronic indirect predator exposure also produces long lasting changes in anxiety and defensive behaviors. While the CORT response to chronic restraint stress tends to habituate over time (Magarinos et al. 1995), there was an increase in basal corticosterone levels that did not appear to habituate up to 20 days after the start of daily predator stress (Blanchard et al. 1998). Further, chronically exposed animals demonstrated a blunted corticosterone response to a novel restraint stress.

Rodents, even those that have been bred and raised in a laboratory, show an innate and immediate stress response to odors produced in the urine, hair, and scent glands of their predators. Cat odors alone, using odorants coming from a used collar worn by a cat (Dielenberg and McGregor, 1999), a ball of cat fur (Vazdarjanova et al., 2001), or used cat litter (Cohen, et al., 2004) have also been shown to cause both immediate and long-term changes to behavior commensurate

with those seen after actual predator exposure. These behavioral changes are dependent on many brain regions that are involved in other forms of stress. Lesions or temporary inactivation of BLA have been shown to prevent the expression of anxiety and avoidance during cat odor exposure (Vazdarjanova et al. 2001). Lesions to the medial amygdala, ventral hippocampus, and dorsal premammillary nucleus inhibit predator and predator odor induced defensive behaviors during exposure (Blanchard et al. 2005). Systemic (Blundell et al. 2005) and amygdala specific (Adamec et al. 2005) injections of NMDA receptor antagonists administered before predator exposure block the stress-induced increase in anxiety. Post-stress systemic administration of beta-adrenergic receptor antagonists, mineralocorticoid receptor antagonists (Adamec et al. 2007), a serotonin receptor 2 (5-HT₂) selective antagonist (Adamec et al. 2004), and the SSRI sertraline (Matar et al. 2006) have all demonstrated therapeutic effects on later anxiety behavior, although the extent of their benefits are not consistent in the literature. Future studies may have to target specific regions of the brain in order to understand the mechanism causing these long lasting behavioral changes.

Individual differences in anxiety and the stress response

As mentioned previously, approximately 6.8% of adults in the United States are diagnosed with PTSD in their lifetime (Kessler et al. 2005). Yet, most surveys estimate that as much as 75% of the population reports experiencing at least one traumatic life event; and of those who experience trauma, only 5 to 35% actually develop PTSD and other types of anxiety disorders (Breslau et al. 1998; Breslau 2001; Sledjeski et al. 2008). This is in stark contrast to the way that most animal

models are designed – with a control group and an “experimental” group, and the expectation that the majority of animals will develop anxiety symptoms. This has recently become an important topic of debate in translational research of anxiety and mood disorders (Yehuda et al. 2006; Yehuda et al. 2007), and has raised important questions about investigating individual differences in the stress response.

One way to concentrate the likelihood of obtaining affected individuals is by choosing strains of rats known to have higher baseline anxiety than others. Lewis rats are an inbred strain developed from the Sprague-Dawley. The Lewis rat strain has been implicated as a model for PTSD, as they exhibit greater baseline anxiety behaviors and greater stress-induced increases in anxiety than other strains. Furthermore, Lewis rats have been characterized by their abnormal HPA stress response (Cohen et al. 2006). Lewis rats have normal basal blood corticosterone levels, as compared to their Sprague-Dawley cousins, but have a hypoactive response to stress.

According to studies using Lewis rats by Cohen, et al. (2004; 2006), one week after a cat odor exposure paradigm the majority of animals demonstrated “extreme behavioral responses,” such as never entering the open arms of the EPM, and heightened acoustic startle that did not habituate. Administration of CORT prior to stress brought CORT levels to those found in other strains, and reduced anxiety behavior. Similar effects have been found in humans. An enhanced acoustical startle response is also common in patients with PTSD (Grillon et al. 2009). There is also some evidence that people who produce lower than average levels of cortisol

after a traumatic event may have a higher probability of later developing PTSD (Yehuda et al. 1994; Yehuda et al. 1998; Delahanty et al. 2000; Yehuda 2002). In addition, people with PTSD symptoms have lower than average basal cortisol levels (Yehuda et al. 1994; Heim et al. 2000).

Individual differences can also be examined by dividing animals into groups based on behavior. This can either be accomplished by collecting basal levels of locomotor, anxiety and depressive behaviors and separating animals into groups prior to experimental procedure, or dividing them after the experimental manipulation based on stress-induced individual behavioral responses. Studies of the former have shown that behavior varies greatly between individuals. In Sprague-Dawley rats, different measures of anxiety behavior including open field locomotion and inhibitory avoidance were correlated within individuals across many days of monitoring (Cure et al. 1992). In a longevity study, fear of novelty in infant rats predicted later CORT responses to novelty in adulthood and correlated with shorter lifespan (Cavigelli et al. 2003).

In Wistar rats grouped by the amount of rearing behavior elicited in the open field, high and low rearers were associated with a number of behavioral differences as well as brain differences. High rearers moved around more, but spent less time in the open arms of the EPM and had poorer retention for both passive and active memory tests (Borta et al. 2005). However, they were also quicker at obtaining food reward and resistant to weight loss (Gorisch et al. 2006). This would indicate that some of the traits that would point towards high anxiety may be beneficial for survival in the wild. During novelty exposure, high responders had increased

extracellular dopamine levels compared to low responders in the nucleus accumbens (Saigusa et al. 1999) suggesting that these two groups of animals have differences in their reward-learning pathways. Additionally, high and low responders were differentially affected by the administration of agonists and antagonists to alpha and beta adrenergic receptors suggesting that receptor concentration in the nucleus accumbens correlates with behavioral differences (Tuinstra et al. 2000).

Experiments where individuals are divided into groups based on their stress response provide information about what brain regions mediate stress vulnerability and stress resilience. While there are very few of these types of studies in the literature, there is both structural and molecular evidence of differences between the stress vulnerable and stress resilient. Two weeks after a predator stress, animals that showed low post-stress anxiety had shorter, more compact dendritic branching in the pyramidal neurons of BLA, whereas highly anxious animals looked no different from unstressed controls (Mitra et al. 2009). This would suggest that stress resilience may be an active process. In another study, after predator stress, animals that were found to be affected by stress had decreased levels of brain-derived-neurotrophic-factor (BDNF) in the *cornu Ammonis* region 1 (CA1) of the hippocampus (Kozlovsky et al. 2007). Additionally, only the animals with the highest post-stress anxiety showed decreases in plasma dehydroepiandrosterone (DHEA) levels (Cohen et al. 2007). In stress resilient animals, mRNA expression of the neuropeptide galanin was higher in CA1, while galanin levels were reduced in both

CA1 and PFC of the stress vulnerable. Injection of galanin prior to stress was also able to abolish stress-induced anxiety one week later (Kozlovsky et al. 2009).

In another model of stress resilience, rats that had undergone a chronic mild stress paradigm were divided by whether or not the stressor had produced anhedonia in a sucrose preference test (Bergstrom et al. 2008). Rats that were vulnerable to stress-induced anhedonia had reduced mRNA expression of the neurotrophic factor vascular endothelial growth factor (VEGF) in region CA3 of the hippocampus, while the anhedonia resistant individuals had upregulated expression of BDNF in CA3 (Bergstrom et al. 2008). In another model that investigated individual differences in extinction learning, animals were divided into two groups based on their ability to recall previous extinction learning of a fear-conditioned stimulus. Electrophysiological recordings of cells in the IL region of mPFC revealed that individuals with better extinction recall had more activity in IL during recall, and that activity in IL was dependent on NMDA receptor activity (Burgos-Robles et al. 2007). There is evidence that neuropeptide Y (NPY) plays a role in stress resilience in both animals and humans, which will be discussed further below. As a whole, these results substantiate the recent trend to examine individual differences in the stress response, and provide evidence that changes in brain and behavior are closely correlated.

Modulators of the stress response

Glucocorticoids

Glucocorticoid imbalance has been implicated in a number of disorders. While chronic elevation of cortisol is a common symptom of major depression and Cushing's disease (McEwen 2005); there is evidence that people with lower than average basal cortisol levels are more likely to develop PTSD (Yehuda et al. 1998). Some clinical trials have found success administering cortisol in the hospital to patients undergoing cardiovascular surgery or treatment for septic shock in reducing later occurrences of PTSD (Briegel et al. 1999; Schelling et al. 1999; Schelling et al. 2001; Pitman et al. 2005). It has also been reported that 10mg/day cortisol treatment, considered to be a low dose, can reduce symptoms of chronic PTSD (Aerni et al. 2004). However, even in these studies, only a small number of people reported feeling better, while many others reported no difference (Yehuda 2002).

In rats, low glucocorticoid levels also correlate with likelihood to develop anxiety after stress (Cohen et al. 2006). Injection of corticosterone (CORT) prior to stress prevents stress induced anxiety and reduces the acoustic startle reflex (Cohen et al. 2006). Moreover, glucocorticoid administration has been shown to prevent retrieval of previously created fear memories in rats and in humans (de Quervain et al. 2000; Roozendaal et al. 2004).

However, studies of chronic CORT treatment in rodents show stress-like effects of increased generalized anxiety, enhancement of fear and contextual conditioning (Korte 2001), and impairment of spatial learning in the Morris water maze (Roozendaal 2000). Chronic high-dose glucocorticoid administration in

rodents produces effects on hippocampal dendritic remodeling and memory identical to those seen from chronic stress (Woolley et al. 1990; Wellman 2001; McEwen 2005). Likewise in the BLA, chronic CORT administration induces the same hypertrophy found during stress (Mitra et al. 2008), thus implicating glucocorticoids and their receptors in the mechanism behind stress-induced structural plasticity and anxiety behavior

It is important to note that these effects are sometimes produced by doses that provide higher than physiological hormone levels and that the usual method of systemic injection is inherently aversive and stressful. In contrast to invasive injection studies, there is evidence that noninvasive administration of low doses of corticosterone (CORT) in the drinking water can prevent restraint stress-induced remodeling of dendrites in the CA3 region of the hippocampus (Magarinos et al. 1998). However, either stress or CORT administration alone produced dendritic hypertrophy. In addition, depressive-like behaviors in rats are increased not only by the absence of glucocorticoids through adrenalectomy (Edwards et al. 1990; King et al. 2001) but also by higher than average CORT levels (Kademian et al. 2005).

The variability of these results suggests a non-linear adaptive response to glucocorticoids (Roozendaal 2000), and supports an inverted U-shaped dose-response theory for hormones, stress, and their effect on brain and behavior (McEwen 2001). At very low and very high levels of circulating glucocorticoids, we may speculate that stress is more likely to alter neural plasticity and memory in a negative way, resulting in increased amygdala activity, dendritic remodeling, and non-fear related memory impairment.

Since it appears that glucocorticoids are important molecular modulators of many of the physiological stress effects found in the brain, then it is possible that by controlling glucocorticoid levels, one could control an organism's behavioral stress response and prevent the long-term effects on behavior. Unfortunately, there is no straightforward answer whether to increase or decrease glucocorticoid levels in order to prevent maladaptive stress responses. In order for this research to translate into clinical applications, basal glucocorticoid levels and the hormonal stress response of each patient would have to be well understood in order to make a diagnosis for each individual.

Catecholamines

Along with glucocorticoids, the role of the norepinephrine system in the study of stress has become a very popular topic. While beta-adrenergic receptors do not appear to be necessary for fear conditioning (Lee et al. 2001; Debiec et al. 2004), there is evidence that these receptors are involved in the consolidation of hippocampal-dependent fear memories in both animals and humans. Beta-blockers, such as propranolol, administered prior to contextual conditioning impair learning and subsequent recall of the conditioned response to context (Ji et al. 2003; Grillon et al. 2004; Roozendaal et al. 2004). Based on electrophysiological evidence, blockade of beta-adrenergic receptors in the amygdala is sufficient to inhibit LTP in the hippocampus (Ikegaya et al. 1997), suggesting that the site of beta-adrenergic influence is in the amygdala and not directly on the hippocampus. It is not fully understood how these receptors are modulating the stress response, but activation of the beta-adrenergic receptors may turn on downstream molecular pathways that

along with circulating glucocorticoids, enhance neural plasticity in the hippocampus. Beta receptor blockade prevents glucocorticoid-induced impairment of contextual conditioning and inhibitory avoidance memory (Quirarte et al. 1997; Roozendaal et al. 2004). This evidence further supports the importance of the amygdala in controlling how other brain regions respond to stress, and helps to emphasize the involvement of multiple modulatory neurochemicals systems in different aspects of fear learning.

An important finding regarding propranolol is that it appears to be effective in preventing stress-related hippocampal-dependent memory if administered after stress. Results from early clinical trials although small in number, appeared promising (Pitman et al. 2005), however, recent studies have not found beneficial effects (Stein et al. 2007; McGhee et al. 2009). Another exciting application of this drug may not be in the prevention of PTSD, but in the treatment of already established symptoms. People who experience a traumatic event often are not able or willing to seek immediate help, and it is only after symptoms develop that they seek treatment. There is evidence that long-term fear memories can be abolished during a secondary consolidation, or reconsolidation, after a reminder cue (Sara 2000). In rats, systemic administration of propranolol immediately after re-exposure to a previously conditioned stimulus can block the reconsolidation of the fear memory, thus impairing recall of the conditioned response 24 hours later (Debiec et al. 2004).

Serotonin

Research examining the role of serotonin and serotonin receptors in the stress response has shown potential for PTSD treatment. Several antidepressants, including selective serotonin reuptake inhibitors (SSRIs), have been somewhat successful in modulating the effects of acute and chronic stress in rats. Although SSRIs are often prescribed to patients with PTSD (Friedman 2004), they have produced an interesting paradox in animal models. The SSRI Fluoxetine has been shown to mitigate stress-induced anxiety and weight-loss (Berton et al. 1999) and reverse the stress effects on sucrose preference and glial cell death in the PFC (Banasr et al. 2007). However, the SSRI, citalopram, has been shown to reduce fear conditioning responses only if administered chronically before conditioning, and it may actually enhance conditioning when acutely administered (Burghardt et al. 2004). Other SSRIs, such as paroxetine and sertraline have shown to be more valuable for stress related depression than for stress related anxiety (Ramanathan et al. 2003)

A potential candidate for PTSD treatment is tianeptine, which enhances serotonin uptake, but has a mechanism of action different from SSRIs (McEwen et al. 2005). Tianeptine administration after an acute stress prevents the stress-induced inhibition of LTP in the hippocampus without causing abnormal levels of LTP when administered to unstressed controls (Shakesby et al. 2002). Chronically, tianeptine prevents hippocampal dendritic atrophy when given concurrently with chronic stress, and is able reverse previous stress-induced atrophy when given daily after chronic stress (Magarinos et al. 1999). In contrast, fluoxetine had no

effect on dendritic remodeling, but enhanced neurogenesis in the dentate gyrus (Duman et al. 2001). In the amygdala, tianeptine studies also indicate that it can prevent stress-induced enhancement of anxiety and growth of BLA neurons (McEwen et al. 2004).

There have been only a few clinical trials using tianeptine for PTSD. In one small trial, although patients reported a decrease in symptoms after daily tianeptine administration, this was not significantly different than patients given fluoxetine (Onder et al. 2005). Still, results cited above suggest that tianeptine may be more beneficial at the structural level than SSRIs (McEwen et al. 2005).

Glutamate

There is much evidence that the universal excitatory neurotransmitter glutamate and its receptors are involved in the stress response. The ionotropic glutamate receptor N-methyl D-aspartate (NMDA) has been heavily investigated for its role in memory consolidation, including fear memories. NMDA receptors are both voltage-gated and ion-gated, thus requiring depolarization of both the presynaptic and postsynaptic cell before they are able to open. This is often referred to as coincidence detection, and NMDA receptors are responsible for long term potentiation of activity between multiple cells in a circuit, which is often thought of as the hallmark of neural plasticity (Morris et al. 1990; Shors et al. 1997). NMDA receptor activity is necessary for both fear conditioning acting through the BLA (Maren et al. 1996) and fear extinction acting through the mPFC (Burgos-Robles et al. 2007). Injection of NMDA receptor antagonist into CE or dorsal hippocampus have also been shown to prevent stress-induced anxiety behavior (Adamec 1997;

Padovan et al. 2000). Since modulating NMDA receptors can both impair fear conditioning and enhance extinction learning, this has made NMDA receptor agonists and antagonists a potential therapeutic target for PTSD and anxiety disorders (Heresco-Levy et al. 2002; Ducrocq et al. 2005; Lehner et al. 2009),

The role of small neuropeptides

NPY and its receptors

Neuropeptide Y (NPY) is a small (36 amino acids) peptide that is part of the pancreatic polypeptide family, and the only one found in both the brain and periphery (Berglund et al. 2003). NPY is released only from neurons, and the highest levels are found in the paraventricular nucleus, and arcuate of the hypothalamus, hippocampus and amygdala (Allen et al. 1983; de Quidt et al. 1986). It is often a co-transmitter with norepinephrine (Sheikh et al. 1988; Niu et al. 1996) and it has shown to be involved in many behavioral functions, including feeding, reward, and anxiety. Injection of NPY or NPY receptor agonists in the hypothalamus increase food intake and receptor antagonists decrease food intake (Morley et al. 1987). NPY also has the ability to modulate leptin levels (Wang et al. 1997), while both leptin and insulin production inhibit NPY expression (Sato et al. 2005). There is also evidence that NPY may be neuroprotective. Under control conditions, NPY receptor agonists are anti-epileptic and protect against excitotoxicity in the hippocampus (Xapelli et al. 2006).

Glucocorticoids have been shown to modulate the actions of NPY. An adrenalectomy can prevent NPY administration from causing obesity (Sainsbury et

al. 1997) that glucocorticoids, NPY and the metabolic hormones are all closely linked. Chronic administration of dexamethasone increases NPY expression in the arcuate nucleus (Konno et al. 2008). NPY can also modulate anxiety behavior. Injection of NPY systemically, into the ventricles, or directly into CE has anxiolytic effects (Heilig et al. 1993; Zhang et al. 2009). Stress appears to have a differential effect on NPY expression depending on the length and timing. Acute stress increases NPY in the BLA and hippocampus, whereas chronic stress reduces NPY expression in these regions (Conrad et al. 2000; Sweerts et al. 2001; Sergeyev et al. 2005).

Interestingly, NPY has been implicated in the study of stress resilience and increased NPY levels may enhance resilience from maladaptive behavioral responses. There is evidence that war veterans have higher levels of plasma NPY compared to non-veterans. Additionally, NPY levels predicted the extent of current PTSD symptoms, where those with recently reduced PTSD symptoms had the highest levels of plasma NPY (Yehuda et al. 2006). Special forces personnel trained in torture resistance tactics have also been shown to have higher NPY levels than other military (Morgan et al. 2000). In rats, NPY administration into the ventricles or amygdala prevents stress-induced behaviors such as fear potentiated startle and enhanced anxiety behavior after chronic stress (Gutman et al. 2008; Luo et al. 2008; Fendt et al. 2009; Zhang et al. 2009). In a study specifically aimed at investigating resilience, Sajdyk and colleagues (2008) found that pre-treatment with five days of infusion of NPY into the BLA prevented acute restraint stress induced changes in a social interaction test.

In the brain, there are four known g-protein coupled receptors for the pancreatic polypeptides including NPY: Y1, Y2, Y4 and Y5, and all of them have been implicated in the expression of anxiety-like behavior or the modulation of the response to stress (Thorsell et al. 2002; Painsipp et al. 2008). Activation of Y1 receptor through intraventricular injection of an agonist has shown to be anxiolytic (Heilig et al. 1993), and Y1 receptor activation can modulate glucocorticoid release (Dimitrov et al. 2007). In contrast activation of Y2 receptors produces anxiogenic effects, and blocking Y2 receptors reduces anxiety (Heilig 2004). The Y5 receptor is not as heavily expressed in the brain, and is always expressed in neurons that are also positive for Y1 receptors (Parker et al. 1999). However there are relatively high amounts of Y5 in the hippocampus, a region where all the known NPY receptors are expressed (Parker et al. 1998). Systemic administration of a Y5 antagonist reduced anxiety in a model of chronic unpredictable stress, and reduced depressive-like symptoms in a depression-sensitive rat strain (Walker et al. 2009).

The ability of NPY and its receptors to modulate anxiety behavior and its established role in stress resilience in both human and animal studies makes it a prime candidate for continued investigation into its function in encoding individual differences in anxiety behavior, stress vulnerability and resilience.

CART Neuropeptide

Another recent molecule of interest in the study of stress has been Cocaine-Amphetamine-regulated-transcript (CART). Although CART received its name from early research in reward learning, CART appears to have a role in the stress pathway, and manipulation of CART expression can alter anxiety behavior. CART

is an endogenous neuropeptide with no known receptor, although recent evidence suggests that it may have a g-protein coupled receptor that exerts an inhibitory effect (Lakatos et al. 2005). The rat CART gene is translated into an inactive pro-CART precursor that is cleaved into two biologically active small peptides (89 and 102 amino acids long), CART 66-102 and CART 55-102 (Dominguez 2006; Stein et al. 2006). CART 55-102 antibodies are commercially available, and this was the form that was analyzed in the studies below. Like NPY, CART is most likely a co-transmitter. It has been shown to be located in vesicles of GABAergic cells in the nucleus accumbens, hypothalamus and ventral midbrain (Smith et al. 1997; Dallvechia-Adams et al. 2002; Moragues et al. 2003). As with NPY, much of the research on CART has examined its role in appetitive and reward seeking behavior. CART is involved in food intake, and is modulated by leptin (Lambert et al. 1998; Parent et al. 2000; Hunter et al. 2004). It can be an appetite suppressant when injected (Kristensen et al. 1998), and decreased expression of CART has been found in the hypothalamus with anorexia behavior and increased expression in obesity (Johansen et al. 2000; Rohner-Jeanrenaud et al. 2002). CART works in opposition to NPY in relation to food intake; intraventricular injections of NPY increase food intake, while the additional administration of CART peptide, reduces levels back to normal (Vrang et al. 1999)

In pathways of reward learning, both cocaine and dopamine can modulate CART expression in the Nucleus Accumbens; while CART, in turn, can modulate the production of dopamine in the ventral tegmental region (Jaworski et al. 2006). CART administration into the Nucleus Accumbens directly effects behavior. An

infusion of CART into this region reduced both food and cocaine self-administration and inhibited cocaine induced locomotion (Jaworski et al. 2003; Jaworski et al. 2008).

More recently, CART has been shown to be involved in fear modulated pathways in the brain and can affect the expression of anxiety behavior. CART expression in the hippocampus is regulated by glucocorticoids. Adrenalectomy decreases CART mRNA expression in the dentate gyrus of the hippocampus, and chronic corticosterone replacement returns CART expression to control levels (Hunter et al. 2007). In contrast with NPY, CART produces anxiogenic effects on EPM behavior when injected into the ventricles (Chaki et al. 2003) and also increased anxiety behavior when injected directly into the amygdala (Dandekar et al. 2008; Dandekar et al. 2009). Stress induced CART mRNA expression has been shown in several regions, and this appears to be dependent on the duration of the stressor (whether acute or chronic) and the area examined. Hunter and colleagues (2007) found increased mRNA expression in the CE immediately after a two-hour acute restraint stress but no difference between controls after a 21-day chronic restraint stress. In contrast, there was increased CART expression in DG after the chronic restraint stress but no effect was seen at the 2-hour timepoint.

These results suggest that CART may play a modulatory role in the stress response and encode for stress timing, modality or other aspects such as salience of the stress. As CART can directly enhance anxiety, this makes it a good candidate for continued investigation into its function in encoding individual differences in anxiety behavior and stress vulnerability.

Current Aims

The purpose of this research was to determine how stress hormones and neuropeptides modulate the cell-signaling pathways that regulate the stress-induced morphological changes in the brain. Based on the individual differences in emotionality and anxiety found in both experimental and control animals, the goals of this thesis were reshaped to investigate whether these structural and molecular changes correlate with individual differences in general anxiety and the stress response, particularly in an animal model of PTSD. The following studies have been broken down into chapters that attempt to address these aims.

Chapter 3: Small neuropeptides present different profiles based on stressor timing and modality. Here, our objective was to investigate how the brain encodes for and responds to stressors of varying length and different modalities. Using animals that were exposed to stressors that differed in duration, stressor type, and timing of exposures, we examined mRNA expression of the candidate peptides CART, NPY and the Y5 receptor in regions responsible for memory, including fear memories and fear extinction, and reward learning.

Chapter 4: The structural and molecular correlates of individual differences in control anxiety profiles. Humans produce a wide range of anxiety behaviors based on genetic makeup and a lifetime of experience. Similarly, rats elicit anxiety behaviors that also vary greatly. Our objective was to find differences already present by adulthood in the brains of animals that vary in their general anxiety

response. We did this by creating selection criteria to consistently choose animals on the far ends of the spectrum of rat anxiety behavior. We then investigated the influence of these individual differences on the neuronal structure of cells in mPFC and on mRNA expression of the candidate peptides, CART, NPY and Y5 receptor in regions known to be involved in plasticity, learning and memory, and previously shown to be affected by stress.

Chapter 5: Individual differences in an animal model of Post-Traumatic Stress

Disorder. As only a small percentage of humans exposed to traumatic events develop PTSD, we created a stress model where some, but not all animals develop anxiety symptoms after stress. Using the multimodal stress model, we then designed criteria to detect those animals that actually change in their anxiety behavior due to the stress experience and those that do not appear affected by the stressor. Our objective was to investigate how individual differences in the stress response (stress vulnerable compared to stress resilient) changed mRNA expression of the candidate peptides, CART, NPY and Y5 receptor, as well as CART protein levels. The different molecular profiles found between the PTSD-like and Resilient groups validate the multimodal stress model of PTSD and our selection criteria for separating out these groups.

Chapter 6: Mechanisms for stress resilience and prevention of PTSD. In this chapter our objective was to find pharmacological manipulations that would tip the balance of post-stress behavior toward a more resilient phenotype. We examined the efficacy of several proteins and hormones of interest, Corticosterone, NMDA receptors, and Y5 receptors in enhancing stress resilience in our model of PTSD and in a more robust chronic stressor

Chapter 7: General Discussion and Implications for Stress Research. Here we discuss the key findings of the previous chapters, and propose several ways that the peptide CART maybe acting in the brain to reduce anxiety and prevent post-stress PTSD-like symptoms. Future directions for investing CART as a possible therapeutic target in the study of stress disorders is also discussed.

Chapter 2: Materials and Methods

Animals Three strains of adult male rats were used in the following experiments as noted: Lewis, Fischer (F344), and Sprague-Dawley. All rats were obtained from Charles River Labs (Wilmington, MA) and were approximately two months old (weighing 175-250g) at the start of the studies. Upon arrival, rats were housed in either groups of two or three for all experiments and were given one to two weeks to habituate to the animal facility prior to the start of the experiment. Animals were kept on a 12 hour/12 hour light/dark cycle and had unlimited access to chow and water except during experimental manipulations. Experiments occurred during the lights-on period, except as noted. All animals used in this study were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory animals and protocols were approved by the Institutional Animal Care and Use Committee at The Rockefeller University (protocols #04073 and #07092).

Stress. Three types of stressors were used in the following studies:

Restraint Stress. For the restraint stress procedure, a rat was placed in a flexible wire mesh tube with the diameter of about the animal's body size. The ends of the tube were closed off and the restrainer with the rat was returned to the home cage. Animals remained in the restrainers for six hours during the light period, after which they were released into their home cages. This procedure was used only in the chronic stress experiments and was repeated once a day for 21 consecutive days (chronic restraint stress or CRS).

Immobilization Stress. For the immobilization stress procedure, rats were placed in flexible plastic cone-shaped bags typically used for blood draws or decapitation (Decapicones; Braintree Scientific, Braintree, MA). Cones were custom made for animals weighing less than 300g. Once the rat entered, the back opening of the cone was taped closed, while the front end of the cone was left open so that the animal had a source of fresh air. The immobilized rats were then returned to their home cage and remained for two hours during the light period, after which they were released back into their home cage. This procedure was used both as an acute and chronic stressor. In the acute immobilization stress experiments, rats were restrained once using this method, followed by ten days of rest in their home cage (Acute Immobilization Stress plus 10 days rest – AIS+10d). In the chronic immobilization stress experiments, this procedure was repeated once a day for 10 consecutive days (10-day Chronic Immobilization Stress – CIS)

Cat Odor Exposure Stress. For the cat odor exposure procedure, two sources of cat odor (fur and urine) were collected and presented together. Cat fur was collected from outside sources (healthy indoor female cats, with up-to-date shots and regular veterinary visits) and stored in airtight plastic containers. Used organic cat litter was filtered for fecal matter, stored in airtight containers and refrigerated for up to 48 hours. Without the presence of fecal matter, the risk of the rats developing toxoplasmosis was negligible. For animals in the stress group, used cat litter was spread over the bottom of a clean plastic rat cage and a small ball (approximately

4cm in diameter) of cat fur was placed in one corner of the cage. For animals in the control group, unused cat litter was scattered over the bottom of a clean rat cage and a similar size ball of synthetic nylon fur was placed in one corner. Rats were individually placed in the prepared cages for 10 minutes. Behavior was recorded using video cameras. All cat products were sealed in air tight containers and further sealed in plastic bags before entry into the animal facility. Cat products were kept in the fume hood before use and were disposed as biohazard materials, and all used cages and equipment were disinfected with a bleach solution before removal to cage wash facility. All cat odor experiments were performed at or near the hoods or backdraft tables to reduce the spread of odorant throughout the rodent rooms. Stress and control exposures were conducted in two different testing rooms, and all personal protective equipment (gloves, coats, booties) were changed before entering each room in order to prevent transfer of odor into the control rooms. In one experiment, rats were acutely exposed to the cat odor followed by 10 days of rest in the home cage (cat odor stress plus 10 days of rest – COS + 10d).

Multimodal Stress. For the multimodal stress procedure, rats were subjected to a 40-minute acute stressor that combined the 10-minute cat odor exposure immediately followed by 30 minutes of immobilization stress, both as described above. Rats in the control group were exposed to the control cages for 10 minutes and then were returned to their home cages, which remained in the testing room for 30 minutes before returning to the housing room. Rats in these experiments were allowed to rest in their home cages for seven days before further testing or sacrifice.

Blood collection

Tail blood collection. In some instances during immobilization stress, tail blood was obtained from immobilized animals. The tail was cleaned with ethanol, and then a fresh single-edge razor was used to nick the tip of the tail. Blood was collected in heparin-coated 1.5ml tubes and immediately placed on ice. For repeated tail blood collection, the resulting scab was nicked again. Three to five drops of blood (approximately 100-250 μ l) were collected each time, for a maximum of three times. Blood was centrifuged at 1500g for 15 minutes, and the plasma collected and stored at -20°C for steroid hormone analysis.

Trunk blood collection. After rapid decapitation, trunk blood was collected in 7ml EDTA coated tubes using a fresh plastic funnel, and immediately placed on ice. Tubes were spun in a centrifuge at 1500g for 15 minutes, and the plasma collected and stored at -20°C for steroid hormone analysis.

Corticosterone Assay. Plasma was thawed and samples were processed in duplicate using a radioimmunoassay kit (RIA) for rat corticosterone. When large samples of plasma were available (> 100 μ l), the Coat-a-Count RIA kit was used (Siemens Medical Solutions Diagnostics, Los Angeles, CA). When tail blood or only smaller samples were available, the ImmuChem Double Antibody RIA kit was used (MP Biomedical, Orangeburg, NY).

Behavioral testing. Either before, during, or after completing the stress/experimental procedures, behavior was video-taped and analyzed as described below:

Open Field. Rats were placed in a plexiglass square box (80 cm x 80 cm) with 20 cm sides and allowed to explore freely for five minutes. Movement, including distance traveled, mean velocity, time spent immobile, and time and distance in the center versus perimeter was recorded and analyzed by Ethovision XT tracking software (Noldus Information Technology, Leesburg, VA).

Elevated plus maze (EPM). Rats were placed in an apparatus consisting of two open arms (40 cm x 10 cm with a 0.5 cm border) and two closed arms (40 cm x 10 cm with a 20 cm opaque wall around them). These arms met in the middle to form the shape of a “plus” sign. Rats were placed in a closed arm of the maze and allowed to freely explore for five minutes. Movement including distance traveled, time spent immobile, time spent in the open and closed arms, and number of times animals reached the end of the platform were recorded and analyzed by Ethovision XT tracking software.

Cat odor exposure behavior. During the 10 minute exposure to either the cat-odor cages or the matched control cages, behavior inside the cages was video-taped. Behaviors were quantified and analyzed, including total movement, time spent immobile, and number of approaches toward the ball of fur.

Home cage behavior and social interaction: After stress or control exposure, rats were returned to their home cage. Rats were marked in some experiments, using non-toxic permanent markers. The cages were then videotaped for 1-2 hours. Video was analyzed for instances of individual behaviors, including eating, drinking, self-grooming and sleeping; and social behaviors, including grooming others, group sleeping, and aggressive behaviors such as chasing, boxing, pinning and biting.

Sucrose preference: Animals (housed 3 per cage) were deprived of food and water for 6 hours during the light period of their light cycle. Then two bottles were be returned to the home cage, one with regular tap water and one with tap water containing 1% (w/v) sucrose. Animals were exposed to the bottles for 18 hours, locations of the bottles switched at the 9 hour time point. Total volume based on weight of the water bottles was recorded at the 0, 9 hour and 18 hour time point.

Decapitation: A rodent guillotine was used for sacrifice by decapitation. Brains were removed from the skull and flash frozen on dry ice.

Perfusion: An i.p injection of Sodium Pentobarbital (at an overdose of 150mg/kg in saline) was used for anesthesia prior to perfusion. For brain immunohistochemistry, rats were transcardially perfused first with saline containing sodium heparin (10U/L) and then with 0.1M Phosphate buffered saline containing

4% paraformaldehyde. Brains were then post-fixed in the same solution overnight and processed as described below. For cell-loading, rats were transcardially perfused first with 0.1M Phosphate buffered saline (PBS) containing 1% paraformaldehyde and then 0.1M PBS containing 4% paraformaldehyde and 0.125% glutaraldehyde. Brains were then post-fixed for 4-6 hours in the same solution and then transferred to 0.1M PBS prior to sectioning.

Cell-loading: Tissue used for cell-loading was sectioned at 200-250 μ m in 0.1M cold PBS using a vibratome (Leica, Bannockburn, IL). The iontophoretic cell loading procedure performed as described by Radley et al. (2006). Briefly, neurons in layer II/III of the Prelimbic (PL) and Infralimbic (IL) region of the medial prefrontal cortex (mPFC) were iontophoretically injected with of 5% Lucifer Yellow (Molecular Probes, Eugene, OR) in 0.01M PBS. Sections were chosen from both right and left hemisphere and PL and IL regions were identified based on atlas landmarks in an area spanning +3.3 to +2.8mm relative to bregma (Paxinos and Watson, 1998). After loading, sections were coverslipped using PermaFluor mounting medium (Thermo Scientific, Waltham, MA).

Cell tracing and analysis: Filled cells were identified and reconstructed in three-dimensions using Neurolucida software (MicroBrightField, Williston, VT). Cells were chosen based on the following criteria: cells must be pyramidal in morphology and located in layer II/III of the PL or IL with the apical dendrite projecting towards the medial line of the mPFC; dendritic arbors must completely

filled as evidenced by well-defined endings at or near the pial surface; cells will be considered reasonably intact as long as all tertiary branches appear to be complete. Basal branches were traced and considered reasonably intact when at least one tertiary branch was present.

Cells were analyzed for total dendritic material, number of branch points and number of endings for both apical and basal dendrites using Neurolucida Explorer. A Sholl analysis was also performed on the apical dendrite in the Explorer program. Briefly, in Sholl analysis, a series of concentric spheres (30 μ m apart) are placed from the cell body outward. Dendritic material that falls within each distance range (0-30 μ m, 30-60 μ m, etc...) is quantified and number of branch points analyzed. Six to nine cells were analyzed for each animal for PL. As the IL is smaller, four to six cells were analyzed for each animal. For total dendritic material, branch points, and endings, an average was determined for each animal for the apical dendrite. For basal dendrites, the average of all basal dendrites was taken for each cell before further analysis. A student's t-test was used to compare means of different groups. For Sholl analysis, a mean was determined for dendritic material at each distance for each animal, and a repeated measures ANOVA with post hoc analysis (Bonferroni corrected) was used.

Immunohistochemistry: Paraformaldehyde perfused brains were placed in 30% sucrose in buffer and allowed to sink overnight. Sucrose brains were flash frozen in dry ice and stored at -80° C before sectioning at 40 μ m via microtome. Sections were stored at -20° in cryoprotectant [30% sucrose, 30% ethylene glycol in

0.1M sodium phosphate buffered saline (PBS)]. Immunohistochemical analysis was performed on matched sections containing the hippocampus, amygdala and prefrontal cortex using the CART (55-102) anti-rat antibody (1:50,000, Phoenix Pharmaceuticals) by the avidin:biotinylated complex - nickel 3,3'-Diaminobenzidine (ABC-NiDAB) immunohistochemical method described by Hoffman and colleagues (2008). An antibody titration (1:100-1:300,000) was first set-up to find the optimum concentration for the primary antibody. Briefly, chosen sections were removed and rinsed 6 x 10 minutes in 0.05M potassium-phosphate buffered saline (KPBS) at RT. Sections were then incubated in 1% hydrogen peroxide in 0.05M KPBS to remove residual peroxidases, and then rinsed several times in 0.05M KPBS. Sections were then incubated in primary antibody (1:50,000) in 0.05M KPBS containing 0.4% Triton X-100, for 1 hr at RT, then for 48 hr at 4°C. After 48 hrs, sections were rinsed 10x 6mins in 0.05M KPBS and then incubated for 1 hr with biotinylated anti-rabbit secondary antibody (1:600, Vector, Burlingame, CA) in 0.05M KPBS containing 0.4% Triton X-100. Sections were then rinsed 5x 10mins and then incubated for one hour in ABC solution (Vectastain Elite ABC kit). Sections were then rinsed 3x 5 mins in 0.1M sodium acetate and then incubated for exactly 20 minutes in 0.1M sodium acetate containing 2.5% Nickel-sulfate and 0.01% DAB. Sections were immediately rinsed in 0.1M sodium acetate and then rinsed 3x 5mins in fresh sodium acetate solution. Sections were then transferred to 0.1M sodium phosphate buffer, mounted on electrostatically charged slides (Plus slides, Fisher Scientific) and allowed to dry overnight. Slides were then washed in progressively higher

concentrations of ethanol, transferred to 100% xylenes and then coverslipped using DPX (Sigma) mounting medium.

In situ hybridization: Frozen sections used for *In situ* hybridization were cut at 20 µm on a cryostat and placed on Fisher Biotech ProbeOn Plus slides (Fisher, Pittsburgh, PA). Oligonucleotide probes were purchased from Integrated DNA Technologies (Coralville, IA). Oligonucleotide probe sequences were determined from those used in the literature. For each probe, the complement was also purchased tested on several slides as a control. Probes sequences are as follows:

Cocaine-amphetamine-regulated-transcript (CART): The CART oligonucleotide probe used in the following experiments was determined previously in our lab (Hunter et al. 2007), and the sequence used was complementary to nucleotides 223-270 of the rat

CART gene: 5'-ATC GGA ATG CGT TTA CTC TTG AGC TTC TTC AGG-3'.

Neuropeptide Y (NPY): The sequence for NPY was obtained from the literature (Conrad et al. 2000). In order to enhance sensitivity, two probe sequences were used for NPY as a 1:1 cocktail. The first sequence was complementary to nucleotides 1355–1386 of the rat NPY gene: 5'-TGC CCG GAC CTG GCC CCT CTG CTC CGC CCC AT -3'. The second sequence was complementary to nucleotides 1629–1669 of the rat NPY gene: 5'- GCT GGC GCG TCC TCG CCC

GGA TTG TCC GGC TTG GAG GGG TA-3' Both sense and antisense probes were generated and tested through the *in situ* hybridization procedure.

NPY, Y5 receptor (Y5): The sequence for the Y5 receptor was obtained from the literature (Durkin et al. 2000). The following sequence complementary to nucleotides 1086-1130 of the rat NPY Y5 gene was used: 5'-AGA CAC AGG CCG TCT TCT TGC TGT ACC TCC TTC TGT GCT TTC TGA-3'. Both sense and antisense probes were generated and tested through the *in situ* hybridization procedure.

Following the guidelines listed in the terminal deoxynucleotidyl transferase instruction booklet (Promega Corp, Madison, WI) oligonucleotide sequences were incubated with the enclosed buffer, [³³P]dATP (Perkin Elmer Inc., Waltham, MA) and the terminal transferase enzyme in water for 1hr at 37°C. The probe was then purified using the QIAquick nucleotide removal kit (QIAGEN, Valencia, CA) and tested in a Liquid Scintillation Analyzer (Packard Instrument Co., Meriden, CT) to determine efficacy of the tailing reaction. The *in situ* hybridization procedure was adapted from Romeo, et al., (Romeo et al. 2007) Briefly, sections mounted on slides were fixed in 3.7% formaldehyde 5 minutes, then rinsed in phosphate-buffered saline (PBS), followed by triethanolamine-HCl (TEA) with 0.5% acetic anhydride in water. Slides were then rinsed in 2X sodium chloride citrate (SCC) and dehydrated in progressively higher concentrations of ethanol, (70, 95 and 100%), delipidated in chloroform, and rinsed in 100% alcohol. Slides were exposed to pre-hybridization

buffer without probe (150 µl/slide), covered with parafilm and placed in a humidity chamber and incubated at 42°C for 1 h. Slides were then washed in 2× SCC and then 70% and 95% ethanol, and allowed to dry. Slides were then exposed to hybridization buffer (150 µl/slide) with the ³³P-labeled probes (approximately 5 × 10⁵ cpm/slide), covered with parafilm, and placed in a humidity chamber at 42°C overnight. The following day, parafilm was removed and slides were washed in 1X SCC buffer at 55°C, 3x for 15 minutes each. Slides were then transferred to a final 1X SSC wash, and allowed to cool to RT. Slides were then washed in 50% and 90% ethanol containing ammonium acetate, followed by 100% ethanol. Slides were air dried for 1 h and then exposed to Kodak BioMax MR film for 2-4 weeks to generate autoradiographs.

Densitometry analysis: Densitometry analysis was conducted on both autoradiographs in the case of the *in situ* hybridization studies and on NiDAB mounted sections for immunohistochemical studies. Films or slides were placed on a light box with a camera mounted above it. Relative optical density (ROD) measurements were obtained of regions of interest using the program MCID 5.0 (Imaging Research, St. Catharine's, ON, Canada) by comparing the density of the regions of interest subtracted by a background measurement for the same animal. When available, regions of interest on both right and left hemispheres were analyzed and averaged. Regions of interest were identified based on landmarks delineated in The Rat Brain in Stereotaxic Coordinates, fourth edition (Paxinos et al. 1998). Further explanation of the regions of interest examined for densitometry can

be found in Appendix 1. Depending on the number of variable being compared, either a student's t-test or a one or two-way ANOVA was used to compare means of different groups. Post-hoc analyses (Tukey-Kramer or Bonferroni) were also conducted.

Corticosterone modulation of stress resilience: In this experiment, the drinking water was supplemented with 400µg/ml of corticosterone (CORT) in 2.4% ethanol in water. Animals received either the corticosterone supplement or 2.4% ethanol only (vehicle) in their normal water bottles for the 12 hours (overnight) prior to acute multimodal stress. Adult male Lewis rats were divided into four groups: control/vehicle, control/CORT, stress/vehicle, and stress/CORT.

NMDA-receptor modulation of stress resilience: In this experiment, the drug CGP43487, an antagonist for N-methyl-D-aspartic acid receptors (NMDAR Ant), was injected 1 hour prior to the acute multimodal stress or control exposure. Adult male Lewis rats were divided into 6 groups: control/no injection, control/vehicle, control/NMDAR Ant, stress/no injection, stress/vehicle, stress NMDAR Ant. Animals in the injection groups were either injected (s.c in the scruff of the neck) with 5mg/kg CGP43487 (dissolved in ≤ 200µl saline) or with 200µl saline (vehicle). All animals were subjected to a handling procedure that mimicked the injection process (without needle prick) for 5 days prior to actual injection in order to habituate them to the procedure and reduce the stress response from the injection.

Neuropeptide Y, Y5 receptor experiment: In this experiment, the diet was supplemented with a Y5 receptor antagonist (Y5 Ant) before and during 21-day CRS. Adult male SD rats were divided into four groups: control/normal chow, control/ Y5Ant, stress/normal chow, stress/ Y5 Ant. For animals receiving the Y5 Ant, the NPY, Y5 Receptor Antagonist Lu AA33810 [N-[[trans-4-[(4,5-Dihydro[1]benzothiepine[5,4-d]thiazol-2-yl)amino]cyclohexyl]methyl]-methanesulfonamide] was synthesized at Lundbeck Research USA (Paramus, NJ), and mixed in with Research Diets lab chow to the equivalent of approximately 30 mg/kg/day. Y5Ant chow administration began four days prior to the start of the stress experiment and continued throughout the duration of the experiment. Animals in the normal chow group received the same Research Diets chow without drug beginning four days prior to the start of the stress experiment. All food was available ad libitum. During CRS, control animals were handled at least twice a week.

Chapter 3: Small neuropeptides present different profiles based on stressor timing and modality

Many different stimuli and paradigms have been used in literature to elicit a stress response from the rat. Restraint and immobilization stress, predator exposure and predator odor, as well as footshock, social defeat and many other stressors produce short term increases in stress hormones and long term increases in anxiety and depressive-like behaviors (McEwen et al. 1999; Miller et al. 2006). Although often the hormonal and behavioral output is the same, it is possible that the brain is in some way encoding for the different properties of the stressor. With this in mind, the goals of this experiment were to confirm previous findings in the literature for acute and chronic restraint stress using different types of acute and chronic stressors, and to determine whether the mRNA expression profile of the candidate neuropeptides or receptors changed with any of the properties of the stressor. Expression of the two peptides examined, CART and NPY, have previously been shown to be affected by stress (Conrad et al. 2000; Sweerts et al. 2001; Balkan et al. 2006; Hunter et al. 2007). Here, we examine properties of the stressors that including timing (acute or chronic) or type of stressor (immobilization of predator odor), as well as timing after cessation of stress (immediately after stress or 10 days after stress) to determine if there are any long term changes in post-stress mRNA expression.

Experimental Paradigm

Brain sections from adult male SD rats were used in the following four experimental paradigms. For a detailed description, please refer to the *Methods* section:

CIS – Chronic Immobilization Stress. Restraint in flexible plastic bag for 2 hours for 10 consecutive days (n=6 per group, Stress and Control)

AIS + 10 days – Acute Immobilization Stress. Restraint in flexible plastic bag once for 2 hours followed by a 10 day rest period (n=9 per group, Stress and Control)

COS + 2 hrs – Cat Odor Stress. Placement in a cage containing both fur and urine from a cat for 10 mins followed by 2 hrs of rest (n=9 per group, Stress and Control)

COS +10 days - Placement in a cage containing both fur and urine from a cat for 10 mins followed by 10 days of rest (n=6 per group, Stress and Control)

Prior to sacrifice, all animals except for the COS+2h group were placed in the EPM for five minutes, and percent time spent in the open arms and total open arm entries recorded. Slides containing the following areas were examined; dorsal hippocampus, nucleus accumbens, central and medial amygdala, medial prefrontal cortex, orbital frontal cortex. One slide per animal was processed through the in situ hybridization procedure described in the *Methods* section for both the CART, NPY, and Y5 oligonucleotide probe. Autoradiographs of mRNA expression were examined on the light box and relative optical density measured for each region of interest as compared to background. Densitometry measurements for each region of interest were compared using unpaired student's t-tests.

Results

Behavior

For all experiments, there was no difference between Stress and Controls for percent time spent in the open arms (Figure 1, $p > 0.05$ for all groups) or number of open arm entries ($p > 0.05$ for all groups).

CART neuropeptide

CART mRNA expression differed by both stress timing and modality. All optical density calculations were normalized to matched controls from the same experiment. Optical density measurements for experimental groups are shown as a percent of control (optical density measurements for controls = 1, or 100%). In layer II/III of the prelimbic region of the mPFC (Figure 2A), stress decreased mRNA expression for the CIS group, $t(8) = 2.33$, $p = 0.0481$, and there was a trend for stress to reduce CART expression in the AIS + 10d group, $t(13) = 1.87$, $p = 0.0836$. No difference was found between stress and control in the COS + 10d or COS + 2h groups ($p > 0.10$). No differences were found in the OFC (Figure 2B); however, there was a strong trend towards an increase in expression in the COS + 2h group, $t(16) = 2.10$, $p = 0.0521$.

In the dentate gyrus of the dorsal hippocampus, CART mRNA expression was calculated for the granule cell layer of both upper and lower blades. As normalized expression levels did not differ between upper and lower blades, the mean of the two regions was calculated and plotted in Figure 2C. A significant increase in expression was found in the COS + 10d group, $t(10) = 2.91$, $p = 0.0157$,

and trend towards an increase for the CIS group $t(9) = 2.05$, $p = 0.0707$. No differences were found in the dentate gyrus for either the AIS + 10d or COS +2h group ($p > 0.10$).

CART expression in the medial nucleus of the amygdala produced a similar pattern of expression as the hippocampus; CIS caused a significant increase in CART expression, $t(9) = 3.29$, $p = 0.0093$, while there was a trend for and COS + 10d to also increase expression, $t(10) = 1.83$, $p = 0.0966$ (Figure 2D). No differences were again found in the AIS + 10d or COS +2h group ($p > 0.10$). In the central nucleus of the amygdala, no clear differences were seen between stress and control groups ($p > 0.05$), however there was a trend towards a decrease in expression for AIS + 10d, $t(14) = 1.85$, $p = 0.0853$ (Figure 2E).

The shell and core regions of the nucleus accumbens were also analyzed (Figure 2 F and G, respectively). There were no significant differences found in either area ($p > 0.05$). However, there was a strong trend towards an increase in CART expression in the shell region after CIS, $t(10) = 2.10$, $p = 0.0620$, and a trend towards an increase in the core region in the COS + 10d group, $t(9) = 2.12$, $p = 0.0632$.

Figure 1. Post-stress elevated plus maze behavior. Stress did not alter behavior in the EPM as measured by time spent in the open arm (A) or in the number of open arm entries (B) in any of the groups ($p > 0.05$). The groups analyzed were: Chronic Immobil. (10 days of immobilization stress), Immobil. 10d (acute immobilization stress followed by 10 days of rest), Cat Odor 10d (cat odor exposure followed by 10 days of rest), Cat Odor 2hr (cat odor exposure followed by 2 hours rest). EPM behavior was not analyzed in the cat odor stress with 2hr delay, as animals were sacrificed immediately after stress.

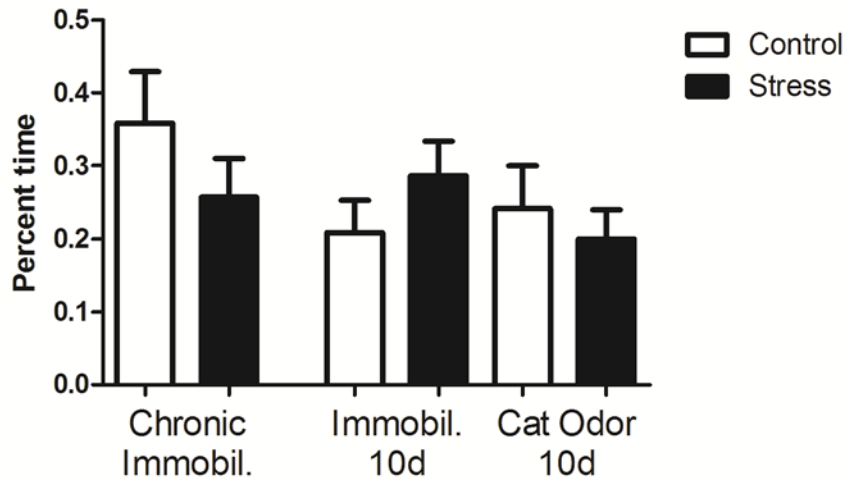
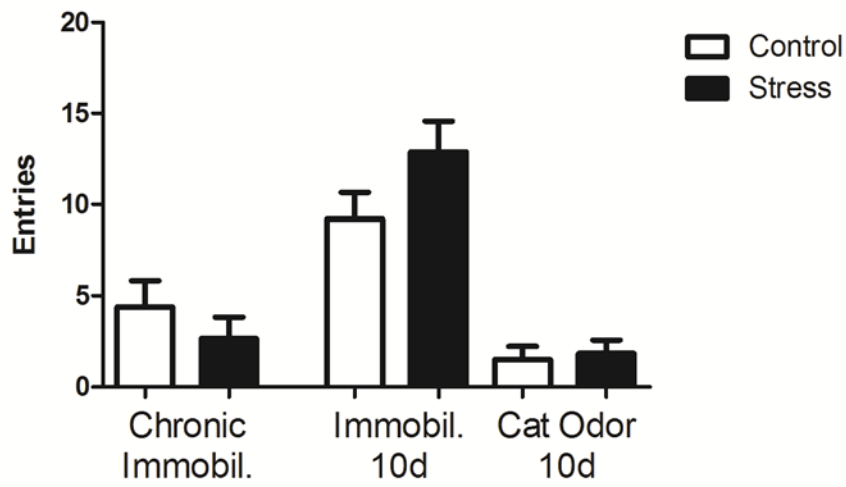
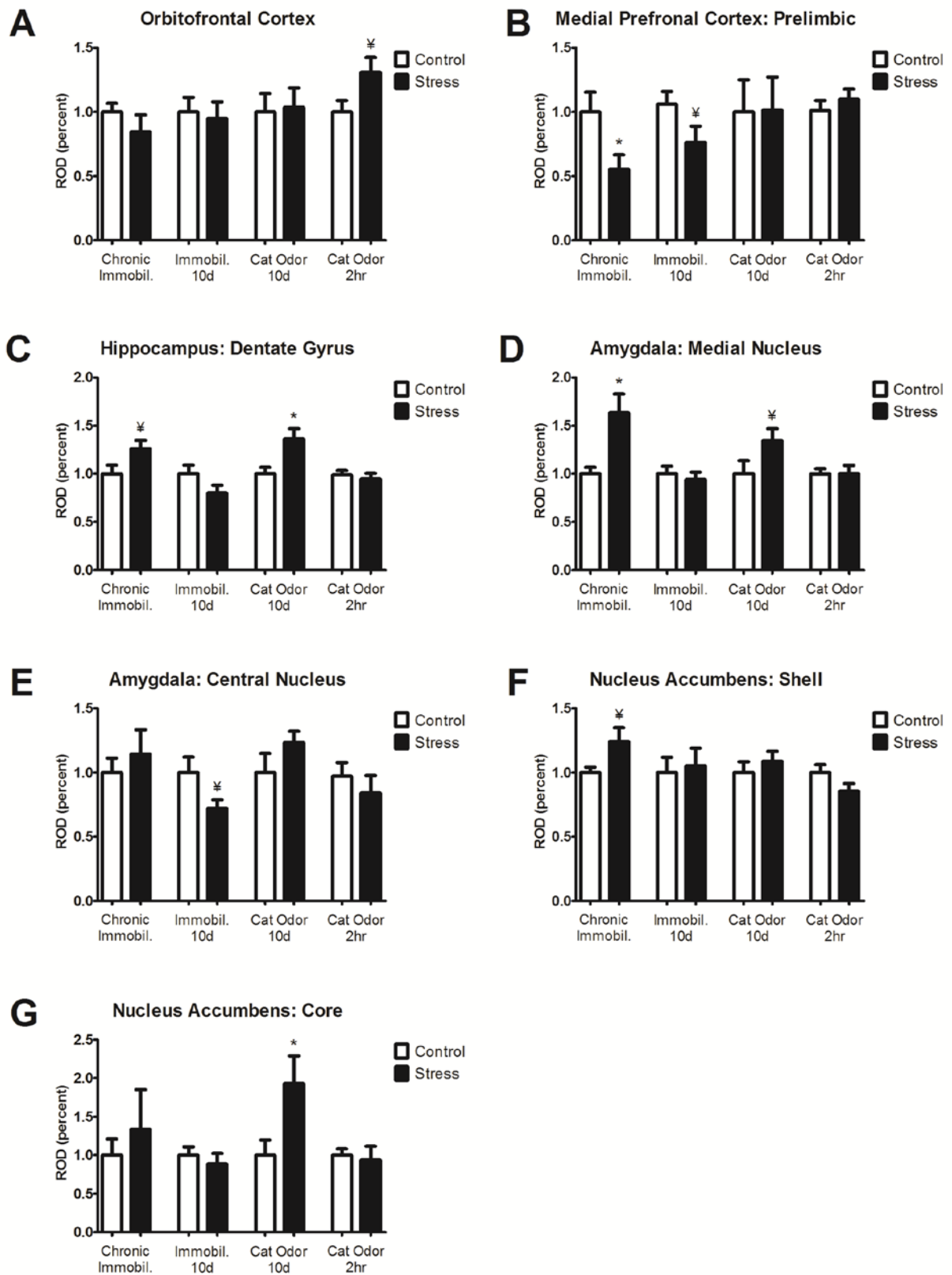
A**Percent time in open arms****B****Entries into open arms**

Figure 2. CART mRNA expression changes with stressor timing and modality. CART mRNA expression was analyzed in four different stress groups: Chronic Immobil. (10 days of immobilization stress), Immobil. 10d (acute immobilization stress followed by 10 days of rest), Cat Odor 10d (cat odor exposure followed by 10 days of rest), Cat Odor 2hr (cat odor exposure followed by 2 hours rest). CART mRNA expression was normalized to controls from the same experiment. Significance is illustrated; *, where $p < 0.05$, and ¥, when $0.10 > p > 0.05$.



Neuropeptide Y (NPY)

NPY mRNA expression differed by both stress timing and modality. All optical density calculations were normalized to matched controls from the same experiment. Optical density measurements for experimental groups are shown as a percent of control (optical density measurements for controls = 1, or 100%). In layer II/III of the prelimbic region of the mPFC (Figure 3A), stress increased expression in the COS + 2h group, $t(16) = 2.33$, $p = 0.0331$. This was the only group to show differences in expression pattern in the mPFC (all other groups, $p > 0.10$).

All other analysis of NPY mRNA expression occurred in the dorsal hippocampus, as this region displayed the highest levels of mRNA expression in the autoradiographs. In the granule cell layer of the dentate gyrus, upper and lower blades were analyzed separately. Again, as there was no difference in pattern of expression, the mean of both blades was calculated and analyzed (Figure 3B). NPY expression was found to increase in the COS + 2h group, $t(16) = 2.19$, $p = 0.0436$. There was a trend towards a decrease in expression in the AIS + 10d group, $t(14) = 1.79$, $p = 0.0950$. No differences were found in the CIS and COS + 10d groups ($p > 0.10$).

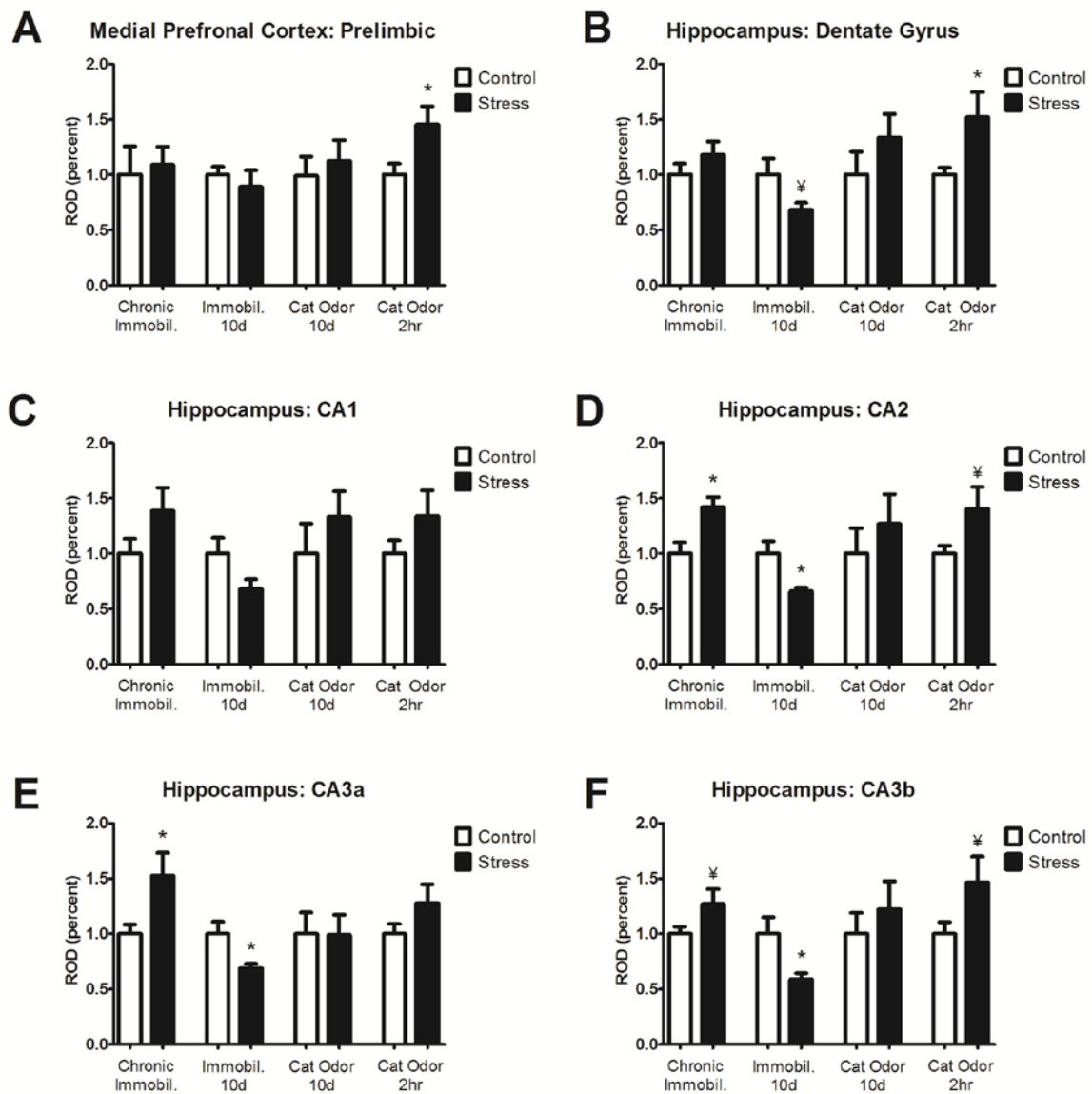
The pyramidal layer of the *Cornu Ammonis* (CA) of the hippocampus was divided into five regions that were calculated separately (see Appendix 1). As there was very low expression of NPY in CA3c, this region was left out of the analysis. In CA1, no stress related differences were found in any of the groups (Figure 3C, $p > 0.10$). This also may have been due to the low levels of expression in this region. In CA2 (Figure 3D), CIS caused a significant increase in NPY expression, $t(9) =$

3.06, $p = 0.0135$. In contrast, there was a significant decrease in expression in the AIS + 10d group, $t(14) = 2.55$, $p = 0.0232$. There was also a trend for stress to induce an increase in expression levels in the COS + 2h group, $t(16) = 1.88$, $p = 0.0782$. No differences were found in the COS + 10d group ($p > 0.1$). NPY expression patterns were very similar in CA3a (Figure 3E) and CA3b (Figure 3F). In CA3a, CIS again increased expression, $t(9) = 2.51$, $p = 0.0334$, while there was only trend for CIS to increase expression in CA3b, $t(9) = 1.87$, $p = 0.0919$. For the AIS + 10d group, stress significantly decreased NPY mRNA levels in CA3a, $t(14) = 2.40$, $p = 0.0307$, and in CA3b, $t(14) = 2.33$, $p = 0.0356$. Although not significant, there was a trend for stress to increase expression in the CA3b for the COS + 2h group, $t(16) = 1.80$, $p = 0.090$. No differences were found in either of these regions for the COS + 10d group ($p > 0.1$).

Figure 3. NPY mRNA expression changes with stressor timing and modality.

NPY mRNA expression was analyzed in four different stress groups: Chronic Restraint (10 days of immobilization stress), Restraint 10d (acute immobilization stress followed by 10 days of rest), Cat Odor 10d (cat odor exposure followed by 10 days of rest), Cat Odor 2hr (cat odor exposure followed by 2 hours rest). NPY mRNA expression was normalized to controls from the same experiment.

Significance is illustrated; *, where $p < 0.05$, and ¥, when $0.10 > p > 0.05$.

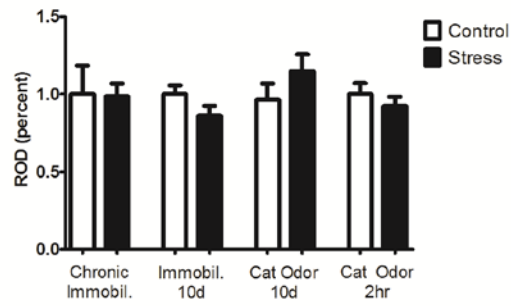


Neuropeptide Y receptor Y5

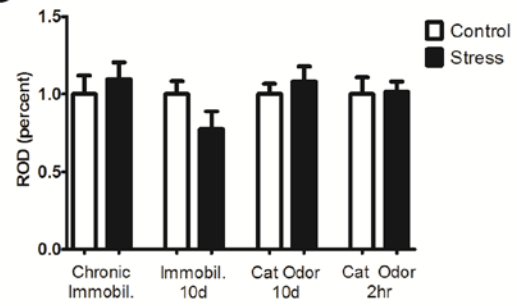
The Y5 receptor oligonucleotide has been shown previous to be effective (Durkin et al. 2000), and Y5 mRNA expression was present in the pyramidal layer of the *Cornu Ammonis* and in the granule cell layer of the dentate gyrus. All optical density calculations were normalized to matched controls from the same experiment. Optical density measurements for experimental groups are shown as a percent of control (optical density measurements for controls = 1, or 100%). In all areas examined, no stress-induced changes to Y5 mRNA expression were found (Figure 4, $p > 0.1$).

Figure 4. Y5 mRNA expression does not change with stress. Y5 mRNA expression was analyzed in four different stress groups: Chronic Restraint (10 days of immobilization stress), Restraint 10d (acute immobilization stress followed by 10 days of rest), Cat Odor 10d (cat odor exposure followed by 10 days of rest), Cat Odor 2hr (cat odor exposure followed by 2 hours rest). Y5 mRNA expression was normalized to controls from the same experiment. For all graphs, the symbol * is used when $p < 0.05$ and ¥ when $0.10 > p > 0.05$. No stress induced differences were found in any of the regions examined.

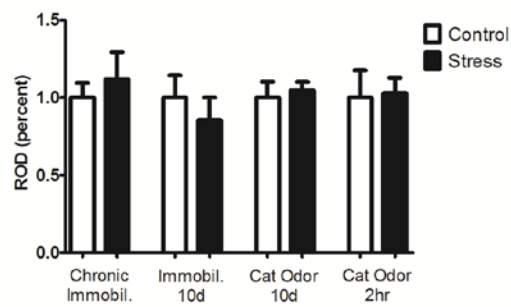
A Medial Prefrontal Cortex: Prelimbic



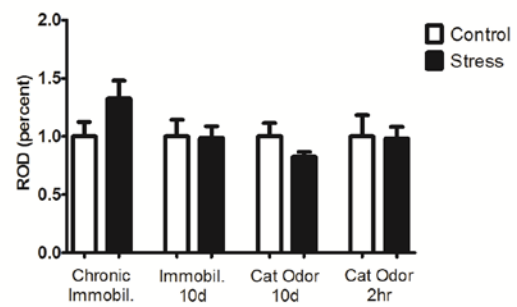
B Hippocampus: Dentate Gyrus



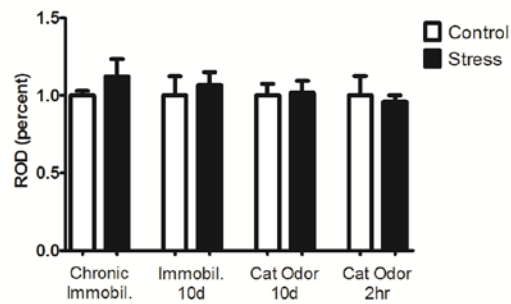
C Hippocampus: CA1



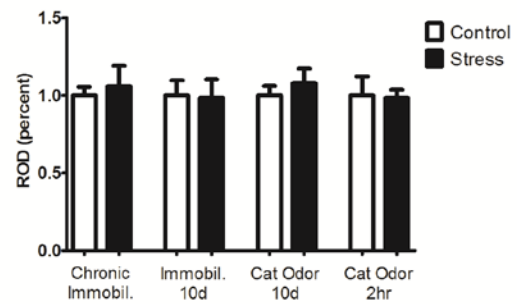
D Hippocampus: CA2



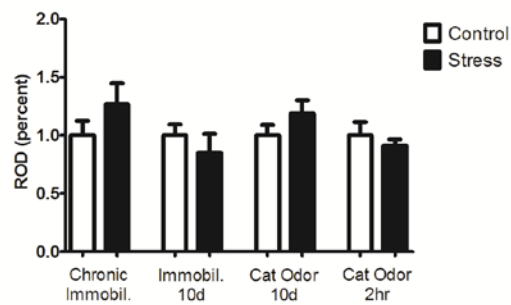
E Hippocampus: CA3a



F Hippocampus: CA3b



G Hippocampus: CA3c



Discussion

The results of these experiments show that there are different molecular profiles in response to stressors that vary by the timing and time course and by the modality, or type of stress. Further, stress appears to cause molecular changes to areas involved in learning and memory and emotional regulation, whether or not there are significant changes to behavior. These results suggest that the brain encodes a stressful experience differently depending on the sensory systems involved and the stressor length and severity, and that the molecular changes are less sensitive to the factors that contribute to the variability in behavior.

The changes found in CART expression were largely in the CIS group. As this was the only chronic stressor that was used in this series, and therefore more severe, it would be expected that this group would have the most significant changes. After CIS, an increase in CART expression was found in the DG, ME and shell of the nucleus accumbens. These results generally agree with the literature. Hunter and colleagues (Hunter et al. 2007) found an increase in CART expression the dentate gyrus after a 21-day chronic restraint stress.

Additionally, injection of CART peptide into the central nucleus of the amygdala has been shown to be anxiogenic (Dandekar et al. 2008), but also has some anti-depressant properties (Dandekar et al. 2009). Here we find a stress-related increase in the medial amygdala, which has not been studied but sends strong input to many of the same regions that the central nucleus innervates, including the bed nucleus stria terminalis, parahippocampal areas, hypothalamus, and midbrain regions involved in stress-induced behaviors (Canteras et al. 1995).

In the nucleus accumbens, increases in CART have been shown to inhibit dopamine-induced locomotive behavior (Jaworski et al. 2008) suggesting the CART is acting on cells with dopamine receptors. However, there is also evidence that CART may be inhibiting GABAergic activity in this region (Shirayama et al. 2006). Most of the GABAergic neurons are spiny neurons that project to the globus pallidus and aid in refining reward learning behaviors. Although not studied, it is possible that the stress-related increase in CART expression alters the reward learning pathways through this circuit.

In contrast to other regions, a decrease in CART expression was found in the mPFC. The cortico-limbic pathways linking the mPFC, hippocampus and amygdala have been well studied, particularly in the fear conditioning and extinction circuit in rodents (Quirk et al. 2008) and in human patients with PTSD (Shin et al. 2006). These studies would suggest opposing activity in the mPFC and amygdala, in which the activity of each region can inhibit the other and differentially affect behavior. Extinction learning requires a strengthening of the inhibitory inputs from mPFC to amygdala, and both animals and humans that have an mPFC activity that is out of balance with the amygdala have heightened fear responses.

Chronic stress also changed NPY mRNA expression in the hippocampus. As with CART mRNA expression, NPY expression increased after CIS. There is some conflicting evidence of NPY levels changing with the timing of stress; stress has been shown to increase expression in the medial nucleus of the amygdala and hippocampus over the first few days of stress (Sweerts et al. 2001), whereas chronic stress for an extended period (3 weeks) has been shown to decrease NPY

expression in the hippocampus (Sergeyev et al. 2005). There is evidence that CART positive neurons are juxtaposed to NPY positive axon terminals in both the hypothalamus and medial amygdala (Broberger 1999), suggesting that changes to NPY expression may directly affect CART expression. As CART and NPY administration appear to have opposite effects on anxiety behavior (Heilig et al. 2002; Chaki et al. 2003), NPY may play a role in inhibiting the release of CART thus reducing anxiety.

The profile of the acute stressors was quite different than both chronic stress and varied by stress modality. Ten days after restraint stress (AIS + 10d) there was an overall trend toward a decrease in CART expression in the mPFC and central nucleus of the amygdala. There was also a decrease in NPY expression in the hippocampus, which was the opposite found for chronic stress at the same timepoint. There are several possible explanations for this. As mentioned above, there is evidence that NPY mRNA expression changes over the time course of stress (Sweerts et al. 2001), and that this is strain dependent. Behaviorally, although neither stress group differed from controls, the animals in the AIS+10d group appear to be slightly less nervous than both the CIS or COS + 10d group. One possibility may be that a single acute restraint stress that is not strong enough to induce anxiety has taught the animal something else, such as resilience. This possibility is considered further in the general discussion.

The other acute stressor, the COS + 10d group, had a completely different profile from AIS + 10d. There was a stronger effect of COS + 10d on CART expression than NPY expression, and the changes in CART expression appear to

be consistent although not entirely overlapping with chronic stress. Similar behavior was also seen in the COS + 10d and CIS group. Interestingly, there was a large increase in CART expression in the dentate gyrus and core of the nucleus accumbens. The core of the nucleus accumbens is involved in associating stimuli with valence and motivational value (Bassareo et al. 2002; Shirayama et al. 2006). It may be that a more environmentally relevant stressor, such as cat odor, had a stronger effect on this region. The failure of COS + 10d to change NPY expression further distinguished this stressor from acute or chronic restraint stress. Although it is not clear why there are no changes to NPY expression, this is further support for the idea that both neuropeptides are involved in encoding the complexity of this stimulus.

The COS + 2h group was the only group that illustrated the immediate changes that occur in mRNA expression for CART and NPY neuropeptides. CART expression appeared to be less affected acutely by cat odor stress than it was in the long term. COS induced an increase in CART expression only in the orbitofrontal cortex. However, there was a consistent increase in NPY expression in the mPFC and hippocampus. This was further evidence that the change in NPY expression is more immediate and the changes in CART are farther downstream; it may be necessary to have longer stress experience or time for consolidation of learning before CART expression changes

Surprisingly, no changes to Y5 receptor mRNA expression were found in any stress manipulation. This would suggest that although there are changes to NPY, this is not affecting the expression Y5 receptor, at least in regions where NPY is

altered by stress. As other NPY receptors, such as Y1, and Y2, are more prevalent in these regions of learning and memory (Parker et al. 1998), it is possible that the changes in NPY levels are acting on these receptors.

In summary, mRNA expression of the neuropeptides CART and NPY are differentially affected by both stress timing and modality. Chronic stress had the greatest effect on CART expression, inducing increases in the hippocampus, amygdala and nucleus accumbens. NPY mRNA expression was more responsive in the immediate aftermath of an acute stress, increasing in the dentate gyrus and CA3 region of the hippocampus as well as the mPFC. As molecular profiles changed in response to stress without changes in behavior, we next wanted to examine the variability and range of behavior in the open field and EPM measures of anxiety. In the following chapter, we examined individual differences in behavioral in response to stress and how that corresponds to the molecular profile of each individual.

Chapter 4: The structural and molecular correlates of individual differences in control anxiety profiles

Individual differences in the behavior of control rats in inbred and outbred strains have been widely reported but few studies have examined structural and molecular changes associated with these differences. The variety of anxiety behavior found in control animals is represented in the wide range of results found in the literature for the open field and EPM. Here, in part because of the variability of behavior described in Chapter 3, we examine the individual differences in control anxiety behavior in two strains of rats, the outbred and commonly used Sprague-Dawley, and an inbred strain known for high anxiety, the Lewis strain. From the results presented in the first part of this chapter, that apical dendritic length of pyramidal neurons in the mPFC correlate with anxiety behavior regardless of stress, and that apical dendritic size in this region has correlated previously with chronic stress-induced deficits in behavior (Liston et al. 2006), individuals from both strains that fell on the extreme ends of the anxiety behavior spectrum were chosen for morphological analysis of pyramidal neurons within the medial prefrontal cortex. Additionally, we examined brain tissue from control individuals selected for repeatedly demonstrating high or low anxiety behavior. Brains were screened for mRNA expression of two neuropeptides previously shown to be altered by stress in Chapter 3 and the literature, CART (Hunter et al. 2007), NPY (Thorsell et al. 1998) and the Y5 receptor (Kakui et al. 2007). We expected that extremely anxious control

individuals would show similar patterns of dendritic remodeling and mRNA expression to that found in stressed animals.

Experimental Paradigm

In the following experiments, unstressed, control animals were divided based on anxiety profile as measured by the open field and EPM. The first task was to establish a consistent way of dividing animals based on their range of anxiety behavior. Then individuals were selected that fit the criteria of “calm” or “anxious,” and their brains examined for both structural and molecular differences

To provide rationale for examining individual differences in the structure of neurons in mPFC, an acute stress experiment was first conducted to determine if a single stress experience could induce changes in dendritic morphology in pyramidal neurons of layer II/III of the PL region. Based on the correlation found between dendritic length and anxiety behavior regardless of stress, individual differences were further investigated. In two separate experiments, 72 Lewis and 60 Sprague-Dawley (SD) used for other experiments (a cohort from the multimodal stress with NMDA receptor antagonist and the chronic restraint stress with Y5 antagonist, respectively; see Chapter 6) were handled for several days prior to behavioral testing and then placed in the open field for five minutes. Time spent in the center and distance traveled in the center of the open field were recorded for each animal. Individuals were selected for the Anxious and Calm groups from an entire population of at least 60 animals, in order to have at least six animals per group that were above and below one standard deviation from the mean. This approximately corresponded to the top 15% and bottom 15% of animals based on time spent and

distance traveled in the center. Six animals (selected from the top and bottom 11 out of 72 for the Lewis rats and top and bottom 9 out of 60 for the SD rats.) were selected from the top 15% and placed in the Calm group and bottom 15% and placed in the Anxious group. Great care was taken to prevent the re-housing of the remaining animals that were used in other experiments. These animals were used for the cell-loading and neuron reconstruction experiment, as described below.

For the molecular profile analysis of anxiety extremes, brain tissue from behaviorally screened control animals sacrificed during previous experiments was used. Lewis rats who served as controls for three separate cat odor stress experiments (multimodal stress as described in Chapter 5, multimodal stress with NMDA receptor antagonist and multimodal stress with CORT in the drinking water, both as described in Chapter 6) were used, as all were exposed to identical and fairly noninvasive experiments. Sectioned frozen brain tissue from these groups that repeatedly fell within the top (Calm) and bottom (Anxious) 20% in two measures of anxiety behavior, the open field and EPM, along with three matched controls per experiment with behaviors that fell near the mean (Average), were processed for *in situ* hybridization.

Experiment 1: Analysis of dendritic arborization of pyramidal neurons in layers II/III of the medial prefrontal cortex after a single acute restraint stress and in control animals with behavioral extremes.

In first part of the experiment, a group of 12 male SD rats (6 controls and 6 stress) underwent two hours of acute immobilization stress followed by 10 days of rest (AIS + 10d). On the tenth day, animals were placed in the EPM and then

immediately sacrificed by perfusion. Sections from the prelimbic region of the medial prefrontal cortex were iontophoretically injected with Lucifer yellow dye as described in the Methods, above. Six to eight neurons from each animal were reconstructed and analyzed.

In the second experiment, 12 SD and 12 Lewis rats were removed from a larger populations based on their open field behavior. Individuals whose behavior fell into the top or bottom 15% were divided into Calm and Anxious groups (6 animals each, based on criteria as described above) and were sacrificed by perfusion. Sections from the prelimbic region of the medial prefrontal cortex were iontophoretically injected with Lucifer yellow dye as described in the *Methods*, above. Six to ten neurons from each animal were reconstructed and analyzed.

Experiment 2: Analysis of mRNA expression of small peptides in control animals with behavioral extremes.

Tissue from control animals was selected based upon behavioral extremes (calm, anxious) along with controls that fell near the median control behavior (average) in the EPM (9 animals per group, resulting in 3 groups: Calm, Anxious, Average). Slides containing the following areas were examined; dorsal hippocampus, central and medial amygdala, medial prefrontal cortex and orbital frontal cortex. One slide per animal containing matched regions of interest was processed through the in situ hybridization procedure described in the *Methods* section for the CART, NPY, and Y5 oligonucleotide probe. Autoradiographs of mRNA expression were examined on a light box and relative optical density measured for each region of interest as compared to background. Densitometry

measurements for each region of interest were compared using a one-way ANOVA. A Pearson's correlation analysis was also performed to compare EPM behavior to densitometry results to examine individual differences.

Results

Time spent in the center of the open field was plotted in a frequency histogram for both the Lewis and SD group (Figure 5A and B). Neither the frequency distribution histogram for the Lewis rats nor SD rats met the requirements for a Gaussian distribution based on the D'Agostino & Pearson omnibus normality test ($K^2 = 16.82$, $p = 0.0002$, and $K^2 = 6.87$ $p = 0.0453$, respectively). Animals selected from the top 15% were placed into the Calm group and animals selected from the bottom 15% were placed into the Anxious group.

Lewis strain controls were analyzed in the open field and seven days later in the EPM. Percent time spent in the center of the open field was plotted against percent time spent in the open arms of the EPM (Figure 5C). A Pearson's analysis found that these behaviors correlated with each other ($r = 0.377$, $n = 36$, $p = 0.0233$). Individuals that fell within the top third of the range in both behaviors were placed in the Calm group, while individuals that fell within the bottom third of the range in both behaviors were placed in the Anxious group. Individuals in the Average group were taken from the center of the range.

Experiment 1

Behavior in the EPM was measured 10 days after AIS treatment. An unpaired student's t-test found no difference in the percent time spent in the open

arms between stress and controls ($p > 0.05$, Figure 6A). Mean apical dendritic length was measured in pyramidal neurons from layers II/III of the mPFC in both groups. An unpaired student's t-test found no differences in apical dendritic material between stress and controls ($p > 0.05$, Figure 6B). However, when total apical material was plotted against EPM behavior for each individual, regardless of stress, a correlation appeared (Figure 6C). Time spent in the open arms of the EPM correlated positively with total apical dendritic length in pyramidal neurons of the prelimbic area of the mPFC (Pearson correlation, $r^2 = 0.520$, $p = 0.0123$). This data was used to support the hypothesis that control individuals selected for high anxiety (Anxious) and low anxiety (Calm) would have morphological differences in pyramidal neurons of the mPFC, as described below.

Figure 5. Factors for separating groups based on anxiety behavior.

Frequency histograms are shown for time spent in the center of the open field for both (A) Sprague-Dawley (SD) and (B) Lewis rat strains. Blue lines indicated the mean, while dashed lines indicate each standard deviation (SD) away from the mean. Data represented by the bars highlighted in the red boxes approximate the animals chosen from the top 15% (Calm) and bottom 15% (Anxious) for each group (n = 60 rats for SD and n = 72 rats for Lewis). (C) A sample behavior scatterplot is shown for control group individuals used in the densitometry analysis. Anxiety behavior correlates on two behavioral experiments, measured seven days apart ($p < 0.05$). Data represented by the symbols highlighted in the red boxes approximate animals selected that fall within the top 20% (Calm) and bottom 20% (Anxious) over the two behavioral measures.

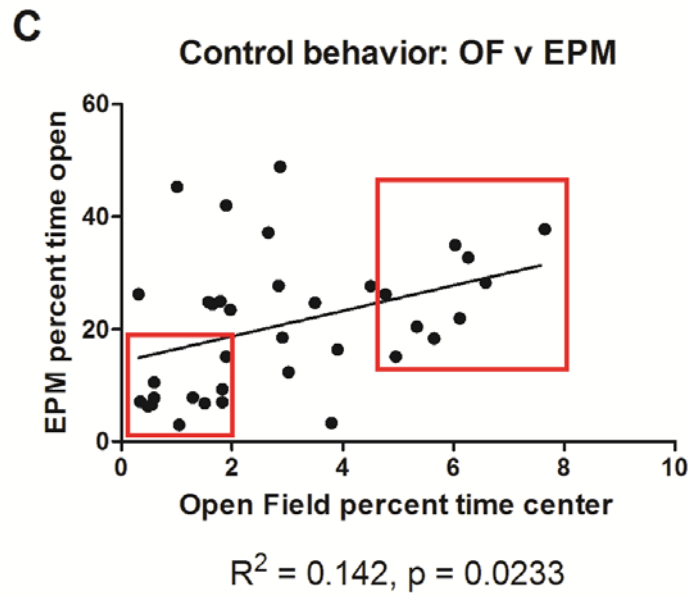
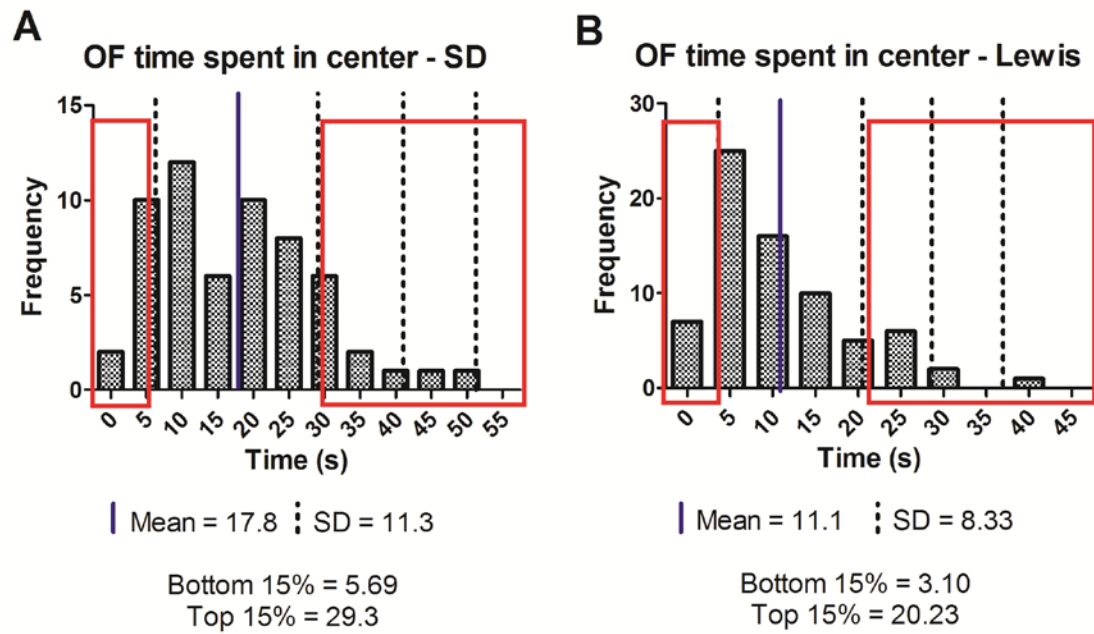
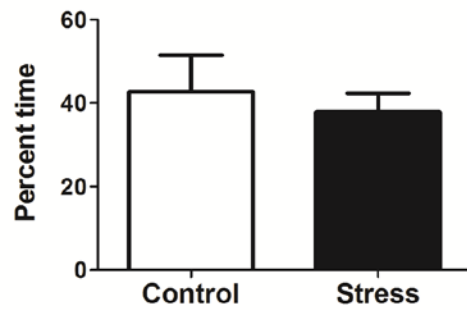
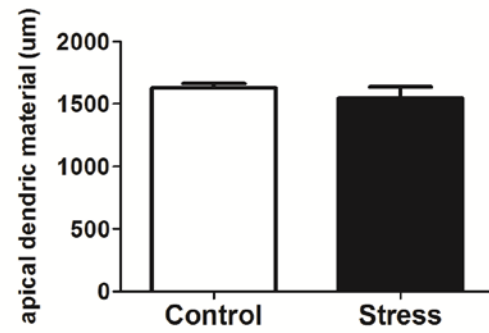
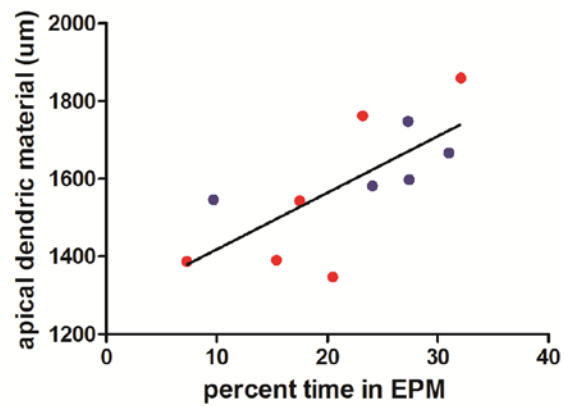


Figure 6. Individual behavior in the elevated plus maze correlated with mPFC dendritic length regardless of stress. (A) No difference was found in behavior between stress and controls 10 days after a two-hour acute immobilization stress ($p > 0.05$). (B) Likewise, no difference was found in total apical dendritic material from pyramidal neurons in layer II/III of the prelimbic region of the mPFC. However, regardless of stress, there was a positive correlation between apical dendritic material and anxiety behavior in the EPM ($R^2 = 0.520$, $p = 0.0123$, red dots are individuals from the stress group, blue dots are individuals from the control group).

A**EPM percent time in open arms****B****mPFC - Apical dendritic length****C****Acute restraint + 10 day
behavior by structure**

Pyramidal neurons from the prelimbic region of the mPFC were chosen from animals that represent the top and bottom 15% of behavior based on time spent in the center of the open field (Figure 5C). As such, an unpaired student's t-test found the animals in the Calm group spent significantly more time in the center of the open field than animals in the Anxious group, in both strains of rats, $t(10) = 8.65$, $p < 0.0001$ for SD, $t(10) = 5.65$, $p = 0.0002$, for Lewis. A two-way ANOVA comparing the time spent in the center of the open field for both Lewis and SD rats in both behavioral groups revealed a main effect of behavior, $F(1,20) = 108$, $p > 0.0001$, as well as a main effect of strain, $F(1,20) = 19.8$, $p = 0.0002$, and a strain by behavior interaction, $F(1,20) = 16.6$, $p = 0.0006$, confirming that Sprague-Dawley rats are generally less anxious in the open field than the Lewis strain (Figure 7).

In the Sprague-Dawley strain, a student's t-test found a larger amount of total apical dendritic material in the prelimbic pyramidal neurons of the calm group as compared to the anxious group, $t(10) = 2.58$, $p = 0.0273$ (Figure 8A); however there was no difference in the number of branch points on the apical dendrite ($p > 0.05$, Figure 8B). Similarly, in the Lewis rat strain, a student's t-test found a larger amount of total apical dendritic material, $t(10) = 3.36$, $p = 0.0072$ (Figure 8C) as well as an increase in the number of branch points in the calm group, $t(10) = 2.77$, $p = 0.198$ (Figure 8D). No effect was found on the basal dendrites ($p > 0.05$ for both strains).

In order to further analyze the differences in dendritic material along the length of the apical arbor, a Sholl analysis was plotted for each behavioral group for both the Sprague-Dawley (Figure 9A) Lewis (Figure 9B) strain. Dendritic material

was calculated into 30 μm bins along the length of the apical dendrite. A two-way repeated measures ANOVA of the Sholl histograms revealed a significant difference in the distribution of apical dendritic material in the prelimbic neurons of Calm and Anxious animals. In the Sprague-Dawley group, this analysis found the expected main effect of distance, demonstrating that the majority of dendritic material can be found within 90-150 μm from the soma, $F(10,100) = 234$, $p < 0.0001$. Additionally, there was an effect of anxiety behavior, $F(1,100) = 7.22$, $p = 0.0249$, and a behavior by distance interaction, $F(10,100) = 3.83$, $p = 0.0002$. Post-hoc comparisons found a significant difference between calm and anxious groups only at the 180 μm point from the cell body ($p < 0.05$, Bonferroni corrected). In the Lewis group, the two-way ANOVA with repeated measures also found the expected main effect of distance, $F(10,100) = 238$, $p < 0.0001$. While there was a main effect of anxiety behavior, $F(1,100) = 5.83$, $p = 0.0364$, there was no behavior by distance interaction, nor any differences at any distance from the cell body ($p > 0.05$ for all comparisons).

Additionally, dendritic length was compared to anxiety behavior for each individual. A Pearson's analysis of dendritic length by anxiety behavior found a significant correlation in the Sprague-Dawley strain, ($r = 0.606$, $n = 12$, $p = 0.0366$, Figure 10A) and in the Lewis strain, ($r = 0.622$, $n = 12$, $p = 0.0309$, Figure 10B). In order to better compare dendritic measurements between strains, the weight of each animal was used as a normalizing factor to compare the relative sizes of the dendritic arbors. As male Lewis rats generally weigh less than the Sprague-Dawley strain at adulthood (see Charles River Laboratories, Inc. pricing and literature guide for animal models), it could be hypothesized that they would also have smaller

brains. This normalizing factor should take into account dendritic length differences based on the size of the brain. A two-way ANOVA of weight-normalized dendritic length scores (calculated by dividing the mean dendritic length by body weight in grams), found a main effect of anxiety behavior, $F(1,20) = 16.4$, $p = 0.0006$, and a main effect of strain, $F(1,20) = 14.7$, $p = 0.001$ (Figure 10C).

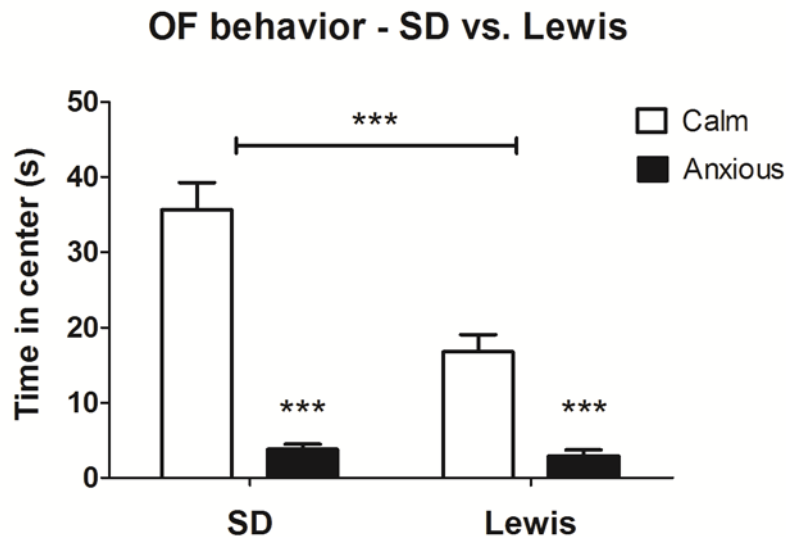


Figure 7. Confirmation of selection criteria for Calm and Anxious groups for both Lewis and Sprague-Dawley (SD) rat strains. In both strains, animals in the Calm groups spend significantly more time in the center of the open field (OF) as compared to those in the Anxious groups ($p < 0.05$). Additionally, there was a main effect of strain, which was driven by the Calm group behavioral data. This suggests that SD rats are generally less anxious than Lewis in the OF, and display a wider range of anxiety behavior than Lewis rats. Significance is illustrated above; ***, where $p < 0.001$.

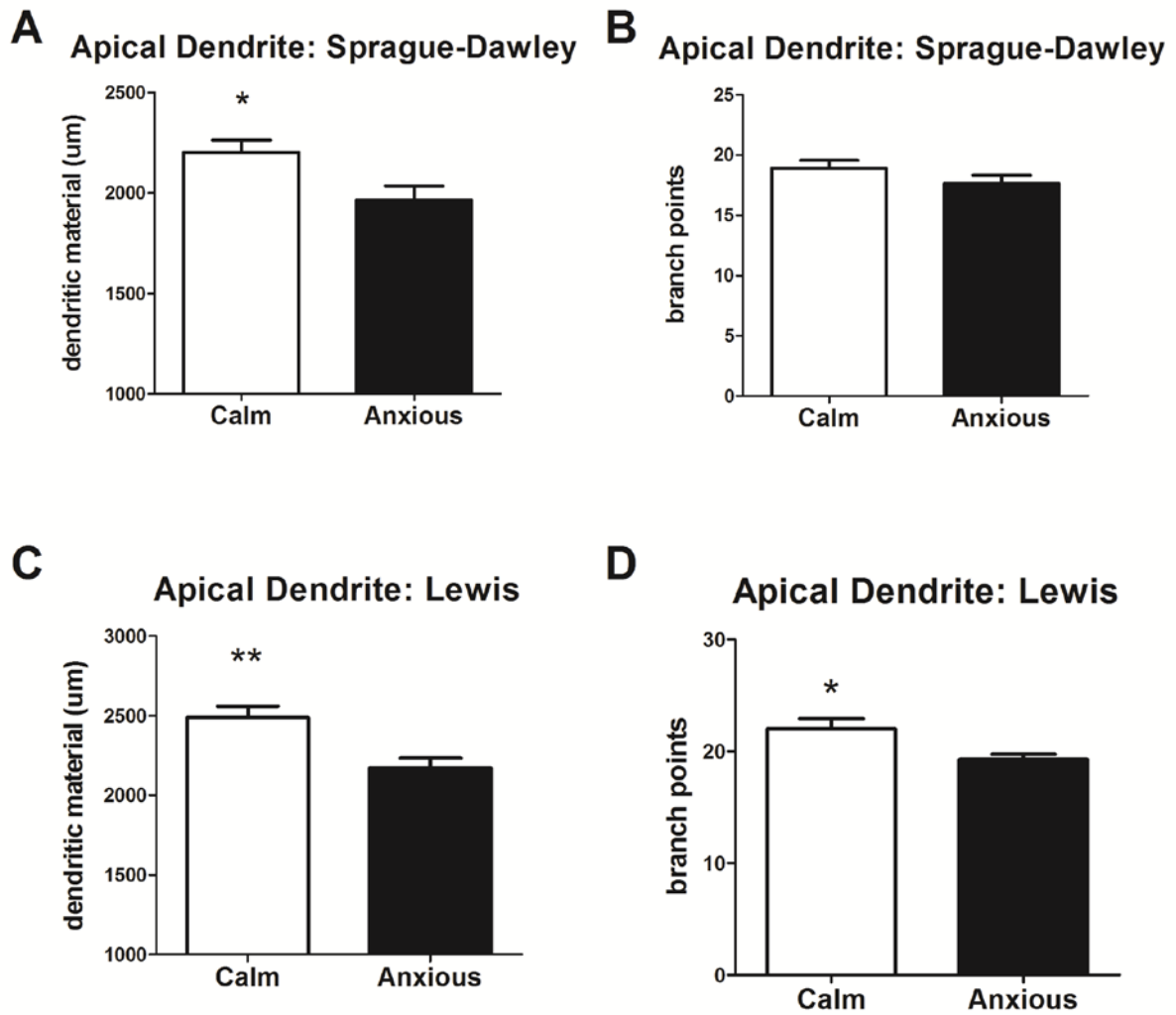


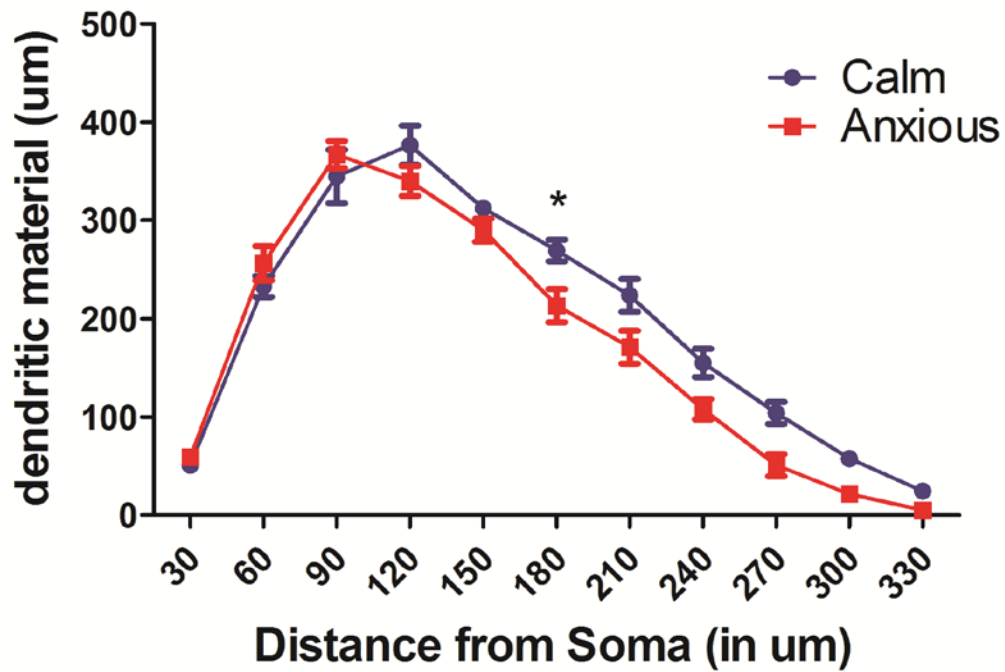
Figure 8. The size of the apical dendritic arbors differ between Calm and Anxious extremes in the prelimbic area of the mPFC in two strains of rats.

Sprague-Dawley rats in the Calm group displayed (A) significantly longer apical dendrites than Anxious group, but no difference was found in (B) the number of branch points on the apical dendrite. Lewis rats in the Calm group had both (A) larger apical dendrites and (B) more branch points on the apical dendrite.

Significance is illustrated above; *, where $p < 0.05$, and **, where $p < 0.01$.

Figure 9. Sholl Analysis reveals overall differences in the distribution of dendritic material along the apical dendrite of pyramidal neurons of the prelimbic area of the mPFC. In (A) the Sprague-Dawley strain, there is a significant effect of behavior ($p < 0.05$) and a significant anxiety behavior by distance interaction ($p < 0.01$), revealing a shift in the concentration of dendritic material in the anxious group. In (B) the Lewis strain, although there is a significant effect of behavior ($p < 0.05$), there is no interaction, suggesting that the increase in apical dendritic material in the Calm group is evenly distributed across the length of the dendrite. Significance is illustrated; *, where $p < 0.05$.

A Sholl Analysis - Sprague-Dawley



B Sholl Analysis - Lewis

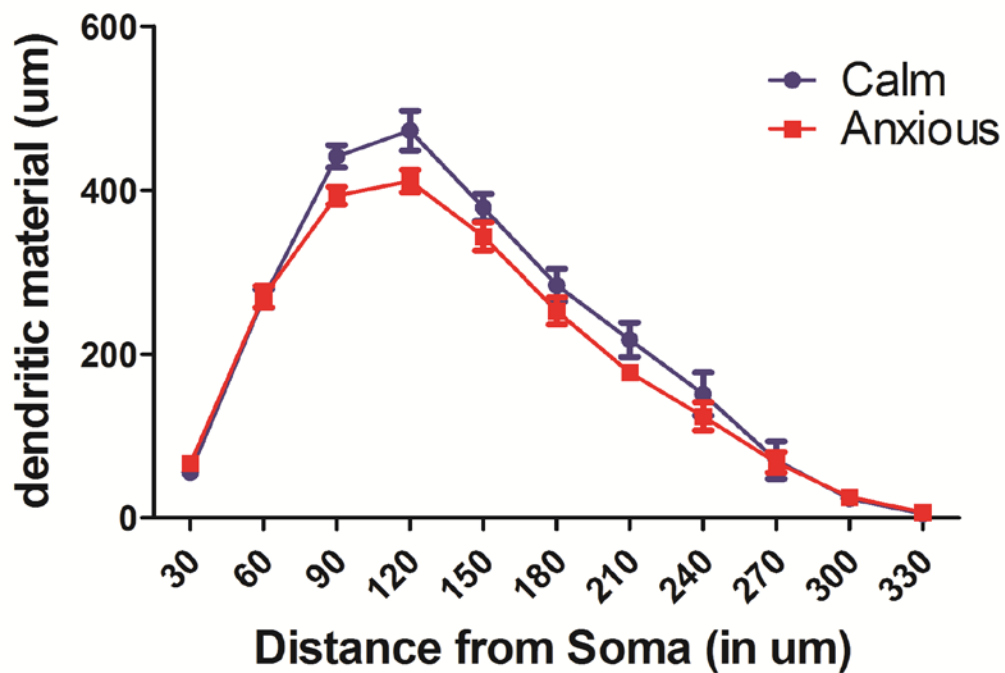
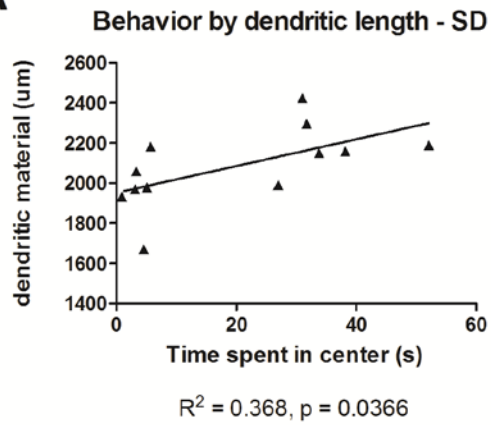
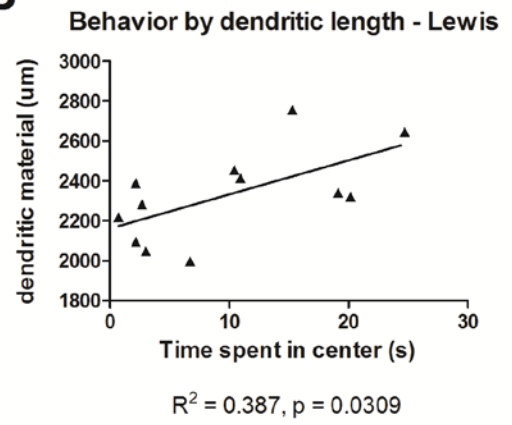
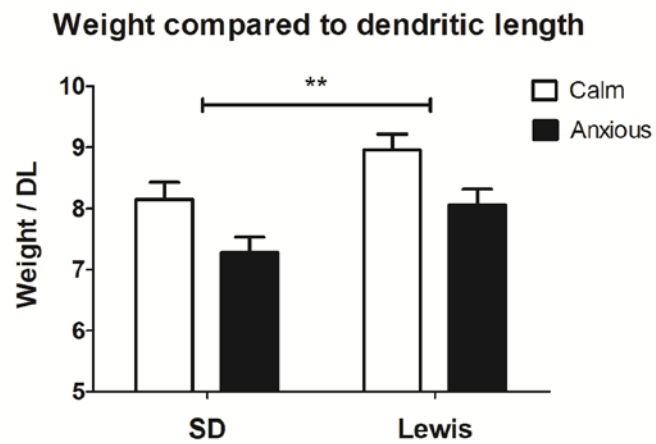


Figure 10. Other factors that contribute to the anxiety behavior and dendritic length differences found in both strains of rats. As individuals were selected based on their anxiety behavior, a significant correlation between dendritic material and open field behavior was present in both (A) the Sprague-Dawley strain, and (B) the Lewis strain ($p < 0.05$ for both analyses). An analysis of body weight as it compares to dendritic length (C) revealed a significant effect of behavior, an also an effect of strain ($p < 0.05$); showing that for their size, the Lewis rats have larger apical dendrites on the pyramidal neurons in mPFC as compared to the Sprague-Dawley strain. Significance is illustrated; **, where $p < 0.01$.

A**B****C**

Experiment 2

As CART and NPY mRNA expression was shown to be differentially expressed during stress based on timing and modality in Chapter 3, expression of the candidate neuropeptides was further analyzed in the control animals divided into groups based on individual differences in anxiety. CART mRNA expression in regions of interest were compared across the three groups of controls as determined by individual anxiety levels; Average, Calm, and Anxious. All optical density calculations were normalized to matched controls from the Average group from the same experiment. Optical density measurements for Anxious and Calm groups are shown as a percent of Average control (optical density measurements for controls = 1, or 100%). In the dorsal hippocampus, both the upper and lower blades of the dentate gyrus (DG) were analyzed separately. In the upper blade of the DG (Figure 11A), a one-way ANOVA showed a significant difference between the three control groups, $F(2, 18) = 3.89$, $p = 0.0383$. Post-hoc comparisons found that individuals in the Calm group had increased optical density compared to Anxious controls ($p < 0.05$, Bonferroni corrected). A one-way ANOVA of the lower blade of the DG (Figure 11B) showed a trend towards a significant difference between the three groups, $F(1,18) = 3.15$ $p = 0.0669$. In the central amygdala (Figure 11C), no differences were found between anxiety groups ($p > 0.05$), however a significant difference was found in the medial amygdala (Figure 11D). There was a significant group effect, $F(2,13) = 5.84$, $p = 0.0155$, as well as a significant difference between the Average and Calm control groups ($p < 0.05$, Bonferroni corrected).

CART mRNA expression was also examined in the prefrontal cortex. A one-way ANOVA comparing control groups found no difference in CART levels in the prelimbic regions of the mPFC (Figure 12A, $p > 0.05$). In the orbitofrontal cortex (Figure 12B) there was a significant group effect, $F(1,18) = 3.93$, $p = 0.0384$. Although there was a trend for the Anxious group to have lower CART expression than both the Calm and Average groups, it was not significant in post-hoc analysis ($p > 0.05$, Bonferroni corrected).

A Pearson's correlation analysis comparing behavior in the EPM to CART mRNA expression in the chosen regions of interest was conducted for all individuals. As all animals in the three anxiety behavior groups were controls, all individuals were grouped into one analysis in order to examine the entire spectrum of control behavior. A positive correlation between CART expression and behavior was found in the dentate gyrus (Figure 13A, $r = 0.456$, $n = 22$, $p = 0.033$), while a negative correlation was found between EPM behavior and CART mRNA expression in the central nucleus of the amygdala (Figure 13B, $r = -0.458$, $n = 23$, $p = 0.0279$). However, no correlation was found between CART mRNA expression and behavior in the medial nucleus of the amygdala (Figure 13C, $r = -0.0245$, $n = 21$, $p > 0.1$). Although not significant, there was a trend towards a positive correlation in the orbitofrontal cortex (Figure 13D, $r = 0.363$, $n = 21$, $p = 0.106$). In the prelimbic region of the mPFC, CART mRNA expression correlated negatively with CART expression (Figure 13E, $r = -0.514$, $n = 18$, $p = 0.029$).

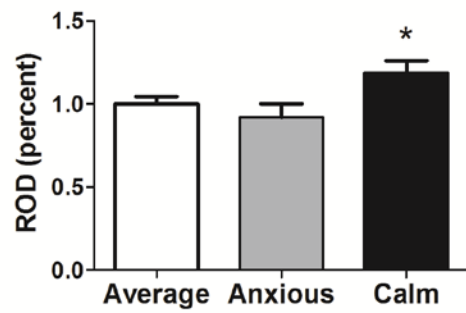
Densitometry for NPY mRNA expression was conducted in the dentate gyrus, upper and lower blade, and CA2, CA3a, CA3b, CA3c as well as the

infralimbic and prelimbic regions of the prefrontal cortex. A one-way ANOVA was conducted between PTSD-like, Resilient and control groups for all regions listed. No differences between groups were found in any region examined. Additionally, a Pearson's analysis was conducted in the same regions, and no correlations between NPY mRNA expression and anxiety behavior were determined ($p > 0.05$ for all groups, data not shown).

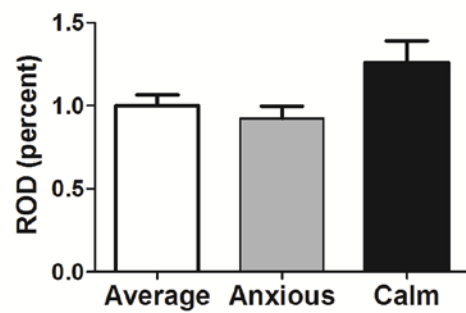
Densitometry for Y5 receptor mRNA expression was conducted in the dentate gyrus, upper and lower blade, and CA1, CA2, CA3a, CA3b, CA3c as well as the infralimbic and prelimbic regions of the prefrontal cortex. A one-way ANOVA was conducted between PTSD-like, Resilient and control groups for all regions listed. No differences between groups were found in any region examined ($p > 0.05$ for all groups, data not shown).

Figure 11. Control groups divided by anxiety behavior differ in CART mRNA expression in the hippocampus and amygdala. There was a significant difference between groups in (A) the upper blade of the dentate gyrus (DG, $p < 0.05$) and, a trend towards a group effect was found in (B) the lower blade of the dentate gyrus (DG, $p < 0.1$). Post-hoc analysis showed the calm group had significantly higher CART expression than the Anxious group in the upper blade ($p < 0.05$, Bonferroni corrected). There was no difference in CART expression levels in (C) the central amygdala. In (D) the medial amygdala a one-way ANOVA found difference between groups ($p < 0.05$). Post-hoc analysis showed the calm group had significantly higher CART expression than the Average group ($p < 0.05$, Bonferroni corrected). Significance is illustrated; *, where $p < 0.05$.

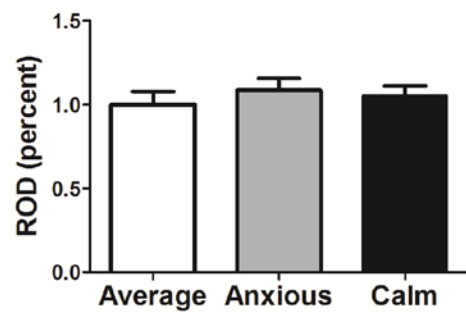
A CART: DG upper blade



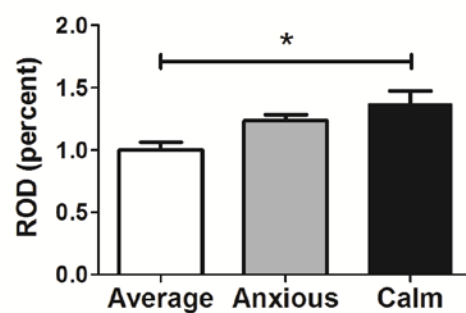
B CART: DG lower blade



C CART: Central Amygdala



D CART: Medial Amygdala



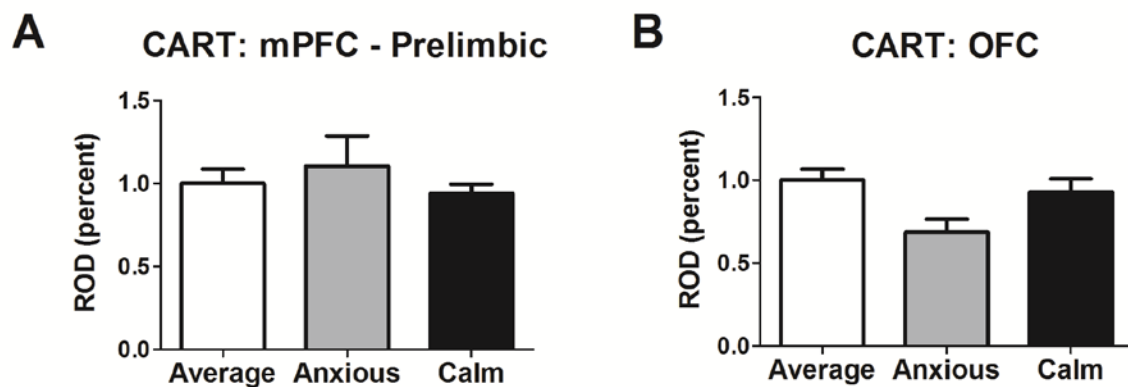
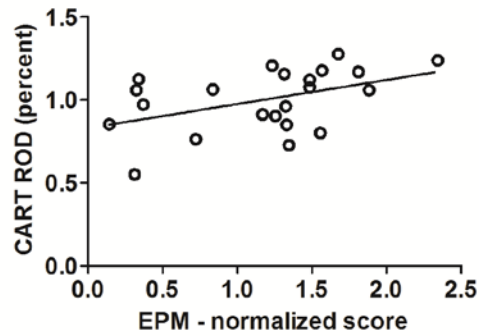
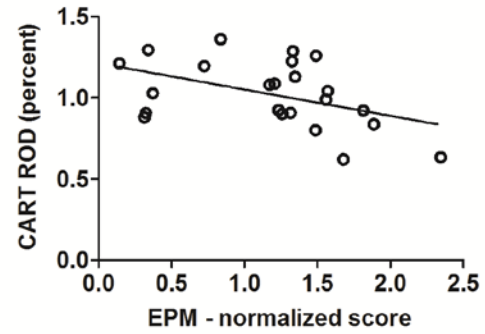


Figure 12. Control groups divided by anxiety behavior differ in CART mRNA expression in the prefrontal cortex. While there is no difference between behavioral groups in (A) the prelimbic region of the medial prefrontal cortex (mPFC, $p > 0.05$), there is a significant group effect ($p < 0.05$) in the orbitofrontal cortex (OFC).

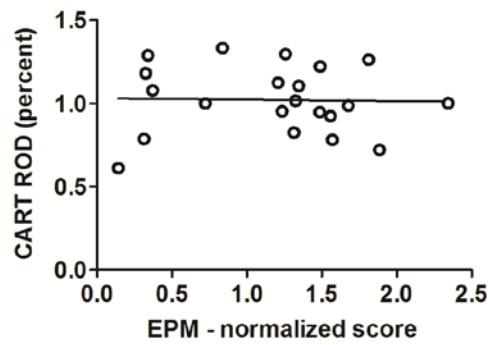
Figure 13. CART mRNA expression correlates with individual anxiety behavior in the elevated plus maze. Probability values can be found under each scatterplot for each region of interest analyzed. A Pearson's correlation analysis found the following; (A) a significant positive correlation in the dentate gyrus of the hippocampus; (B) a significant negative correlation in the central nucleus of the amygdala; (C) no correlation was found in the medial nucleus of the amygdala; (D) a trend towards a positive correlation in the orbitofrontal cortex; and, (E) a significant negative correlation in prelimbic region of the mPFC. For all analyses, significance is achieved when $p < 0.05$.

A**CART expression by behavior
dentate gyrus**

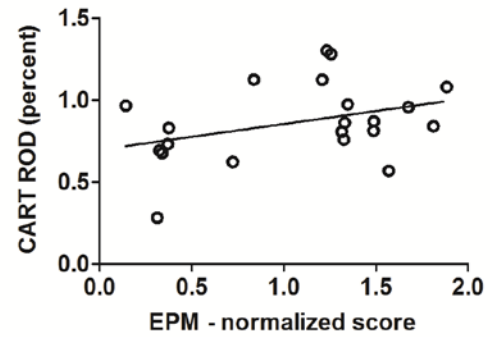
$$R^2 = 0.208, p = 0.033$$

B**CART expression by behavior
central amygdala**

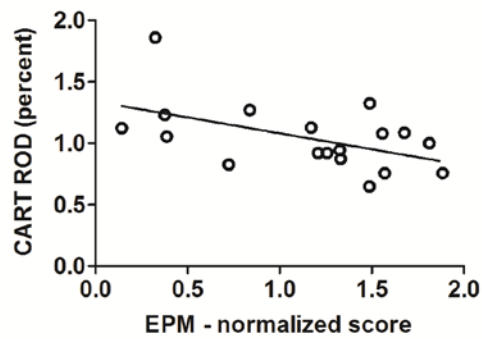
$$R^2 = 0.210, p = 0.0279$$

C**CART expression by behavior
medial amygdala**

$$R^2 < 0.001, p > 0.05$$

D**CART expression by behavior
Orbitofrontal cortex**

$$R^2 = 0.132, p = 0.106$$

E**CART expression by behavior
mPFC - prelimbic**

$$R^2 = 0.265, p = 0.029$$

Discussion

The results of this chapter clearly demonstrate that anxiety behavior varies significantly across individuals that, for all intents and purposes, have identical upbringing, environments, and life experiences, are often siblings and have very similar genetic makeup. This variability in behavior appears to correlate with molecular and structural changes in brain regions known to be highly plastic and involved in learning and memory.

When divided into groups that contained individuals from both ends of the anxiety behavior spectrum, these groups differed from each other in both dendritic arborization in layer II/III of the prelimbic region of the mPFC and in CART mRNA expression profile. Individuals in the low anxiety (Calm) group had significantly longer apical dendrites than the anxious individuals. Although this effect was more robust in the Lewis strain, it was also present in the Sprague-Dawley rats. These effects mirror the same trends seen in mPFC neurons of stressed animals. Three weeks of chronic restraint causes dendritic retraction and an anxious behavioral profile (Cook et al. 2004; Radley et al. 2004; Brown et al. 2005).

Additionally, the Sholl analysis revealed a difference in the distribution of dendritic material between the Lewis and Sprague-Dawley strains. Calm Sprague-Dawley individuals showed a typical distribution of dendritic material across the dendrite, the largest concentration of branches was located around 90-120 μ m from the cell body, and gradually tapered off farther away from the soma. Anxious animals, while having smaller branches in general also had a shift in where the dendritic material was located, with a peak around 90 μ m from the soma and less

material spread out farther away from the cell body as compared to the calm individuals. This difference in distribution may indicate that cells in the prelimbic region of anxious animals are receiving stronger inputs from a different population of neurons, and suggests that there is an overall difference in connectivity in the prefrontal cortex of calm and anxious individuals. Lewis rats in both the Calm and Anxious groups had similar Sholl profiles, suggesting that the increase in dendritic length found in the calm animals was spread across the entire dendritic arbor.

Another interesting result of this research was that Lewis rats have larger apical dendrites compared to body mass on the neurons of layer II/III of the prelimbic cortex when compared to the Sprague-Dawley strain (Figure 10C). This would be in contrast to what would be predicted based on their behavior, since even the calmest Lewis rats spend little time in the open arms of the EPM (Figure 7), which would indicate anxiety. While there is not much published research on strain differences in the prefrontal cortex, Lewis rats appear to have lower c-fos expression after stress (Trneckova et al. 2006).

The differences found in prefrontal cortical dendritic structure between Calm and Anxious controls may account for much of the variability (differences of as much as 15-25% of apical dendrite size) seen in experimental groups after stress. This may be one reason why we have had great difficulty getting a stress effect on behavior and in the brain when subjecting animals to mild and acute stressors, such as those in Chapter 3. The stress-induced changes that are occurring may not be large enough to compensate for the differences that are already present in an adult animal before an experiment has even taken place.

There are many explanations for why there is such a large range of anxiety behavior in control animals that are essentially similar in their genetics and life experiences. The differences that we can't control for include individual genetic variation, which can have a large impact on gene expression (Alfonso et al. 2002), and previous and ongoing social interactions, which includes both maternal care and behavior (Clinton et al. 2007) and social hierarchy in cages in adulthood (Blanchard et al. 1993). Finally, an often disregarded factor that can cause large differences in anxiety behavior is the impact of husbandry procedures or experimenter experiences (Burn et al. 2008). This will be further examined in the main discussion.

Nevertheless, anxiety behavior appears to be consistent across time and across behavioral measures in unstressed animals. This was demonstrated in the correlation found between open field and EPM anxiety measures (Figure 5C). Although not always evident in smaller groups of animals, this trend has been consistent in groups of control animals greater than 24 individuals and in a meta-analysis of all of the previous Lewis controls run in the multimodal stress experiments, normalized by experiment.

CART mRNA expression also differed between Calm and Anxious groups. There was an increase in CART expression in the upper blade dentate gyrus of the calm individuals as compared to the Anxious group. There was also increased expression in the medial amygdala of calm animals as compared to average animals. In the Anxious group, there was a trend towards a decrease in expression

in the orbitofrontal cortex, as evidenced by the significant difference between the three control groups in a one-way ANOVA.

These differences are further explored in the correlation analyses (Figure 13). Notably, CART mRNA expression significantly correlated with behavior in several regions, and not always in the same direction. These correlations with behavior would suggest that if CART mRNA expression is correlated with protein expression in these regions, then CART is either directly influencing the anxiety behavior of the animal, or that a pathway further upstream is acting upon both the CART system and other neurochemical systems to change behavior. Evidence for the former is that injection of CART peptide into the lateral ventricles or central amygdala (Dandekar et al. 2008) can directly enhance anxiety behavior. As we found that increased CART expression correlates with reduced anxiety behavior in another region adjacent to the lateral ventricles, the dentate gyrus of the hippocampus, this would suggest that CART may have opposing effects in different regions. A CART increase in the central amygdala, particularly at higher than physiological levels, may trump the anxiolytic effect that high levels of CART appear to have in the hippocampus. These results indicate that a complex interaction is going on upstream of CART gene expression and behavioral change.

One way to better understand what aspects of the CART expression profile may be induced by stressors that occurred prior to the start of an experiment compared to those that may have more of a genetic component, would be to examine CART expression in stressed animals with different behavioral profiles. From the results in this chapter, it is evident that CART expression varies with

anxiety behavior and disposition before any experimental manipulation has occurred, and it was important that we take that into account. One way to accomplish this is by measuring pre-stress baseline anxiety behavior. We could then select the animals that changed over time (those that develop anxiety due to the experimental manipulations), the PTSD-like, and those who do not change, the stress resilient. In the following chapter, we designed a stress paradigm that creates both of these types of individuals and we investigated differences in their molecular profiles.

Chapter 5: Individual differences in an animal model of Post-Traumatic Stress Disorder

As we demonstrated in Chapter 4 that basal levels of anxiety correlate with both structural and molecular differences in the unstressed brain, we next wanted to confirm that any stressor used to induce anxiety behavior actually caused a change from basal anxiety levels in each individual. Here we adapted and enhanced a published model of Post Traumatic Stress Disorder (PTSD) that used a presentation of cat odor (Cohen et al. 2004). By combining it with another type of stressor, restraint, we were able to increase the number of animals that showed stress-induced behavioral changes. We also added a neutral auditory cue to the stress episode to determine whether presentation of the cue after stress would further enhance anxiety. Similar to human behavior, not every individual showed increased anxiety after stress, some appeared resilient. We then selected for individuals who appeared most affected by the stressor and those whose behavior remained the same, the PTSD-like and the Resilient. Brains from these animals and controls were screened for mRNA and protein expression of two neuropeptides previously shown to be altered by stress in the previous chapters and the literature; CART (Hunter et al. 2007), NPY (Thorsell et al. 1998) and the Y5 receptor (Kakui et al. 2007). We expected that stress-resilient individuals would show similar patterns of mRNA expression to that found in the control animals in the Calm group.

Experimental Paradigm

Following an animal model of Post-Traumatic Stress Disorder (PTSD) previously established in the literature (Cohen et al. 2004), a cat odor stress was implemented that consisted of a 10-minute cat odor exposure using both urine and fur, followed by a seven-day rest period. As no effect on behavior in the EPM was found in either Sprague-Dawley rats ten days after stress (see Chapter 3, Figure 1) or in Lewis rats seven days after stress (data not shown), the multimodal stress paradigm was created in order to develop a longer-lasting stress episode with more contextual cues. For comparison, both Fischer (F344) and Lewis rat strains were tested on the multimodal stressor, which consisted of a 10-minute cat odor or control exposure followed by 30 minutes of immobilization stress. Tail blood was drawn from the stressed rats immediately after cat odor exposure and again 20 minutes after the start of the immobilization stress (30 minutes into stress period) for corticosterone analysis. Blood was not drawn from control animals as this would have created a stressful experience. Seven days after stress or control episodes, anxiety behavior was measured in the EPM. For all other experiments in this chapter, the multimodal stress model was used.

Using the multimodal stress episode with a conditioned cue as a model of PTSD.

In the next experiment 72 Lewis rats underwent the multimodal stress paradigm with the addition of a novel audio cue followed by seven days of rest. Behavior in the open field was measured prior to placing animals in experimental

groups. During the stress or control exposure, animals in half of the groups heard a novel, neutral audio stimulus; a looped two-minute audio track of “Songbirds of the British Wetlands” (British Library online Archival Sound Recordings). The remainder of the animals heard no novel audio cues. There were four exposure groups (18 animals per group, stress/song, stress/no song, control/song, control/no song). On the seventh day after stress or control exposure, animals were placed in the EPM for five minutes. During the five-minute episode, the same audio stimulus was playing in the background for some of the animals, while others heard no additional audio stimuli. All combinations of song exposure and stress were run, although more animals were run in groups where the song would be a relevant cue, played during both exposures [18 stress/song/song(cued), 6 stress/song/no song, 6 stress/no song/song, 6 stress/no song/no song, 18 control/song/song(cued), 6 control/song/no song, 6 control/no song/song, 6 control/no song/no song]. Five minutes after being removed from the EPM, most animals were sacrificed by rapid decapitation and blood collected for plasma corticosterone analysis, although one group (n = 24) was perfused for a different analysis.

Separation of affected individuals by examining changes to anxiety behavior over time

The analysis of the study above was further broken down into behavioral profiles. Animals in the stress groups were divided based on anxiety behavior as measured by the open field and EPM. Difference scores were generated from normalized pre-stress scores subtracted from post-stress scores normalized to the mean of the controls. Stressed individuals with scores well below the control mean

were selected and separated into “PTSD-like” and those near or above the control mean were placed in the “Resilient” group for molecular analysis.

Molecular analysis

For the molecular profile analysis of the PTSD-like and Resilient groups, brain tissue from animals sacrificed during previous experiments were used. Lewis rats that underwent multimodal stress and average controls for three separate experiments (multimodal stress as described above, multimodal stress with NMDA receptor antagonist and multimodal stress with CORT in the drinking water, both as described in Chapter 6) were used. Animals were selected from groups with the fewest other experimental manipulations; however, it was necessary to choose from animals that had been exposed to bird song or 2.4% ethanol in the drinking water for one 12-hour period. Nonetheless, unstressed controls were matched who shared these manipulations.

Sectioned frozen brain tissue from these groups was selected using the criteria described below for PTSD-like and Resilient animals. Slides containing the following areas were examined; dorsal hippocampus, central and medial amygdala, medial prefrontal cortex and orbital frontal cortex. One slide per animal was processed through the *in situ* hybridization procedure described in the *Methods* section for the CART, NPY, and Y5 oligonucleotide probe. Autoradiographs of mRNA expression were examined on a light box and relative optical density measured for each region of interest as compared to background. Densitometry measurements for each region of interest were compared using a one-way ANOVA. For immunohistochemistry and analysis, animals that met the criteria for PTSD-like

and Resilient and matched controls were taken from the perfused sets of tissue collected for the multimodal stress with NMDA receptor antagonist (non-injected groups), described in Chapter 6. Nickel-DAB immunohistochemistry using an antibody against CART (55-102) was conducted on matched brain sections for each individual, and analyzed by densitometry. The hippocampal formation and amygdala were subdivided into layers based on staining patterns and atlas landmarks, and sampled separately.

Results

The multimodal stressor: Lewis vs. Fischer rats

Plasma corticosterone (CORT) levels were calculated from tail blood taken 10 minutes and 30 minutes after the start of the multimodal stressor (Figure 14A). A two-way ANOVA with repeated measures found a significant effect of time, $F(1,13) = 38.6$, $p < 0.0001$, but no effect of rat strain ($p > 0.05$). Anxiety behavior was also compared between strains and stress groups in the EPM seven days after stress or control exposure (Figure 14B). A two-way ANOVA revealed a significant effect of stress, $F(1, 32) = 35.8$, $p < 0.0001$, as well as a main effect of strain, $F(1,32) = 16.4$, $p = 0.0003$. Post-hoc comparisons showed that both Lewis and F344 animals in the stress groups spent significantly less time in the open arms of the EPM ($p < 0.05$ for all analyses, Bonferroni corrected).

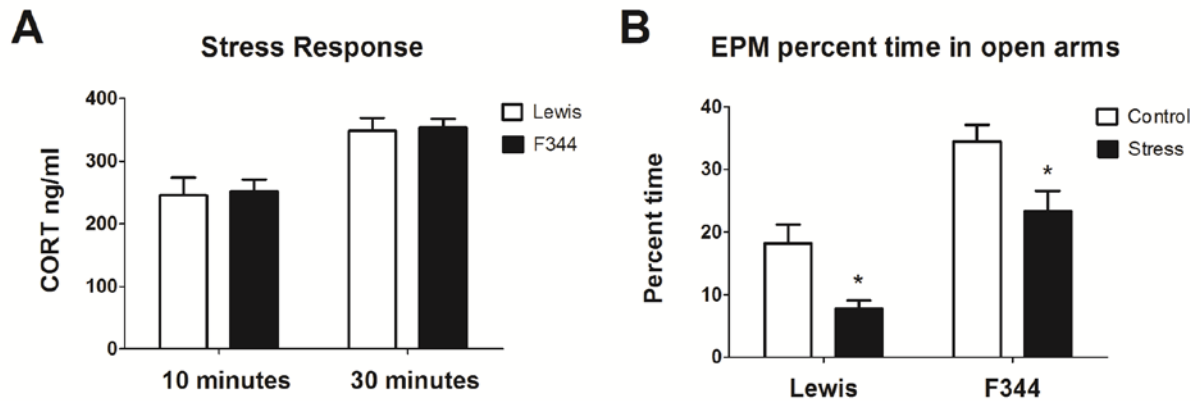


Figure 14. The multimodal stress episode is sufficient for producing a corticosterone response and behavioral changes seven days later. (A) Lewis and F344 rats have similar corticosterone responses to a multimodal stress episode, 10 minutes after the onset of cat odor exposure and 20 minutes after the onset of immobilization stress (30 minute timepoint). (B) Seven days after a single multimodal stress episode, both Lewis and F344 show significant reductions in time spent in the open arms of the EPM, although Lewis rats spend less time in the open arms in general compared to F344. Significance is illustrated; *, where $p < 0.05$.

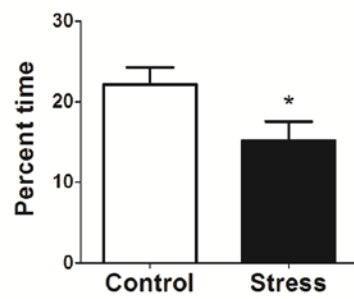
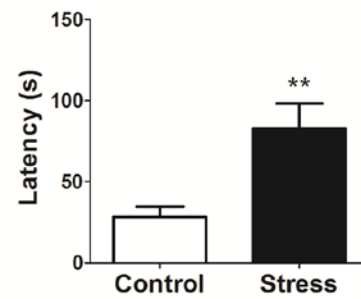
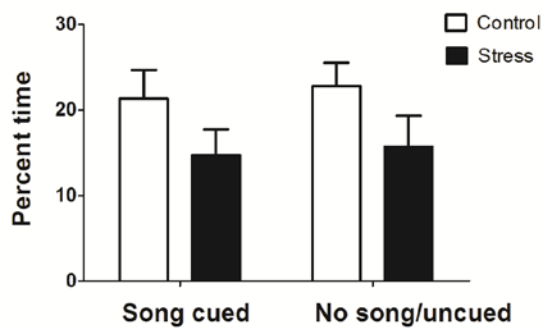
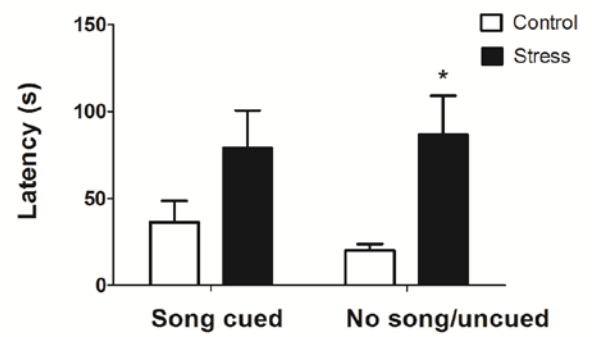
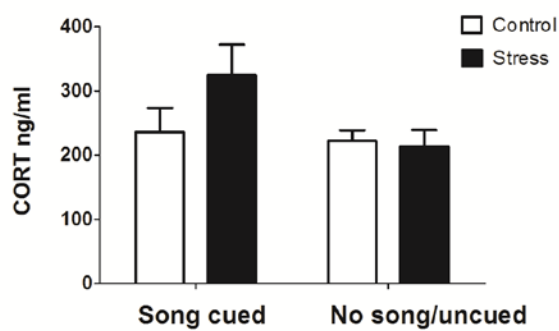
Using the multimodal stress episode with a conditioned cue as a model of PTSD in Lewis rats

For the initial analysis, animals were grouped together by stress or control exposure. Overall, animals in the stress group spent less time in the open arms of the EPM (Figure 15A) and had a longer latency before entering the open arms (Figure 15B) than controls, $t(70) = 2.2$, $p = 0.0316$, and $t(67) = 3.28$, $p = 0.0016$, respectively. Groups were further divided by those who were exposed to the audio stimulus. Of these groups, song would be a relevant cue only when bird song was played during the multimodal stress or control exposure and during the EPM. Therefore, behavior for these animals was separated into a song cued group (either stress/song/song or control/song/song) and a nosong/uncued group (all other groups of audio stimulus exposure) divided by stress (Figure 15C). A two-way ANOVA between stress and controls in the song cued and no song/uncued groups found a main effect of stress on the percent time spent in the open arms of the EPM, $F(1,67) = 4.58$, $p = 0.0362$, however, there was no significant effect of song cue on behavior in the EPM ($p > 0.05$). Analysis of latency to enter the open arms also showed a main effect of stress, $F(1,67) = 10.5$, $p = 0.0018$, but again, no effect of song cue ($p > 0.05$). Post-hoc comparisons found that only the stressed animals in the No song/uncued group differed significantly from controls ($p < 0.05$, Bonferroni corrected).

Plasma corticosterone collected 10 minutes after entry into the EPM was also divided into song cued and no song/uncued groups (Figure 15E). A two-way ANOVA found a main effect of song on corticosterone levels, $F(1,31) = 4.26$, $p =$

0.0474, but no main effect of stress ($p > 0.05$). Post-hoc comparisons were not significant for either the stress or control groups ($p > 0.05$, Bonferroni corrected).

Figure 15. Playing a stress-associated audio cue during behavioral testing in the elevated plus maze does not further enhance the long-term effects of a multimodal stressor. Overall, a multimodal stress episode decreased (A) percent time and increased (B) the latency to enter the open arms of the EPM seven days later. An audio cue played during the multimodal stress or control episode was again played in the EPM in the Song cued group. (C) While there was a main effect of stress on percent time and (D) latency to enter the open arms of the EPM, the presence of the audio cue had no further effect. (E) Corticosterone (CORT) levels 10 minutes after entry into the EPM were higher in the Song cued groups ($p < 0.05$); however, there was no difference between stress and control CORT levels. Significance is illustrated; *, where $p < 0.05$, and **, where $p < 0.01$.

A**EPM percent time in open arms****B****EPM latency to enter open arms****C****EPM percent time in open arms****D****EPM latency to enter open arms****E****Corticosterone after EPM**

Separating out the affected: PTSD-like vs. Resilient behavior

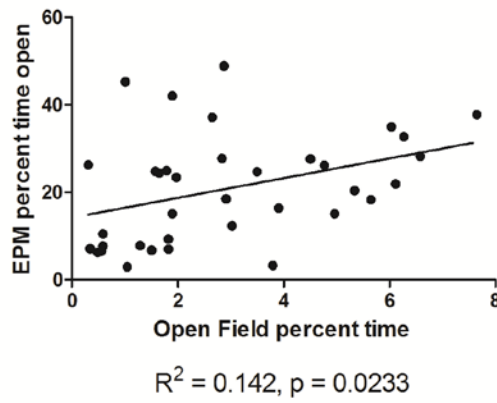
As previously shown, open field anxiety behavior was found to correlate with EPM anxiety behavior measured seven days later (Figure 16A). Pre-stress open field anxiety behavior as measured by percent time spent in the center of the open field was again plotted against post-stress percent time spent in the open arms of the EPM for animals in the stress group (Figure 16B). A Pearson's analysis found that pre-stress and post-stress behavior did not correlate in the stressed group ($r = 0.011$, $n = 36$, $p = 0.542$). As the pre-stress open field test showed a wide range of anxiety levels, a comparison of anxiety behavior was accomplished by calculating z-scores (calculated as percent of the population) for pre-experiment and post-experiment behavior. The pre-experiment z-score was obtained by taking the individual data for time spent in the center of the open field, and subtracting the mean of the entire population. Then this number was then divided by the standard deviation of the entire population, and adjusted to a z-score calculated percentile. The post-experiment z-score was obtained by taking the individual data for time spent in the open arms of the EPM, and subtracting the mean for the Control group, which represents an unchanged population. This was then divided by the standard deviation of the same control group, and adjusted to a z-score percentile. In order to emphasize whether changes from pre-experiment behavior were in the positive (less anxiety) or negative (more anxiety) direction, z-score percentiles for post-experiment behavior were subtracted from pre-experiment behavior to acquire a difference score (Figure 16C). From here, animals that fell above zero (meaning their behavior was unchanged from pre-stress levels) were placed in the Resilient

group, while animals that fell below one standard deviation below the average for the control group were placed in the PTSD-like group.

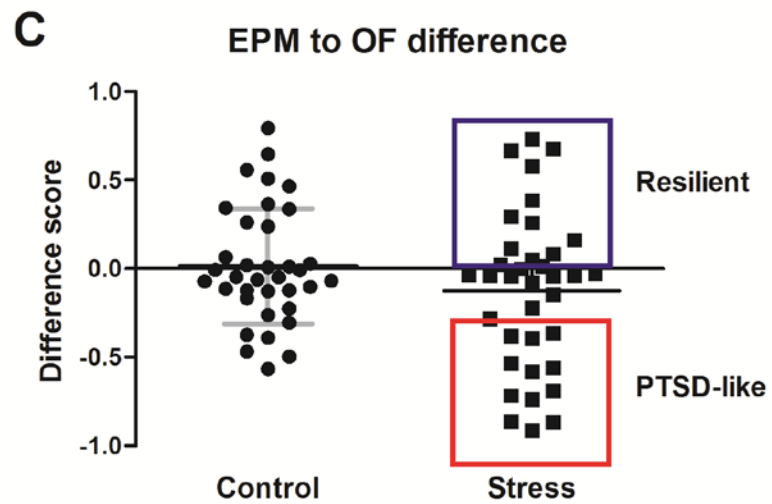
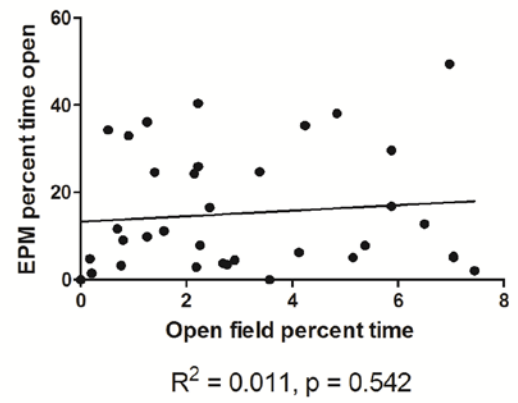
Figure 16. Factors for separating groups based on anxiety behavior. A

scatterplot illustrates the relationship between time spent in the center of the open field and time spent in the open arms of the EPM in the control and multimodal stress groups. (A) A Pearson's analysis determined that anxiety behavior correlates between these two behavioral experiments, measured seven days apart in control animals ($p < 0.05$). (B) However, no correlation was found between anxiety behavior before and after stress ($p > 0.05$). (C) Difference scores were obtained from comparing individual pre-experiment and post-experiment anxiety measures in order to separate animals into those whose behavior had changed over time from those whose behavior remained the same. Data represented by the symbols highlighted in the red box were animals chosen for the PTSD-like group and those in the blue box highlight those chosen for the Resilient group.

A Control behavior: OF vs. EPM



B Stress behavior: OF vs. EPM



CART Neuropeptide molecular profile in PTSD-like and Resilient animals

CART mRNA expression analysis

CART mRNA expression in regions of interest were compared between the PTSD-like and Resilient stress groups along with controls from the Average group (see Chapter 4). All optical density calculations were normalized to matched controls from the Average group from the same experiment. Optical density measurements for PTSD-like and Resilient groups are shown as a percent of Average control (optical density measurements for controls = 1, or 100%). A one-way ANOVA was utilized for comparisons between the three groups, however, the PTSD-like and Resilient stress groups were also analyzed using a unpaired student's t-tests. In the dorsal hippocampus, in both the upper blade (Figure 17A) and lower blade (Figure 17B) of the DG, a one-way ANOVA found an overall difference between groups, $F(2,26) = 5.21$, $p = 0.0125$, and $F(2,26) = 4.82$, $p = 0.0166$. Further analysis revealed that the Resilient group had significantly higher CART expression than the PTSD-like group in both blades ($p < 0.05$). In the central nucleus of the amygdala (Figure 17C), a significant difference between groups was also discovered, $F(2,27) = 3.41$, $p = 0.0471$. A student's t-test found that CART mRNA expression was significantly higher in the Resilient group ($p < 0.05$). No differences were found in the medial amygdala (Figure 17D, $p > 0.05$).

CART mRNA expression was also examined in the prefrontal cortex. A one-way ANOVA showed a significant group effect in the prelimbic region of the mPFC (Figure 18A), $F(2,25) = 3.59$, $p = 0.0427$. Further analysis found that animals in the

resilient group had significantly lower levels of expression than PTSD-like animals ($p < 0.05$). No effect was found in the orbitofrontal cortex ($p > 0.05$, Figure 18B).

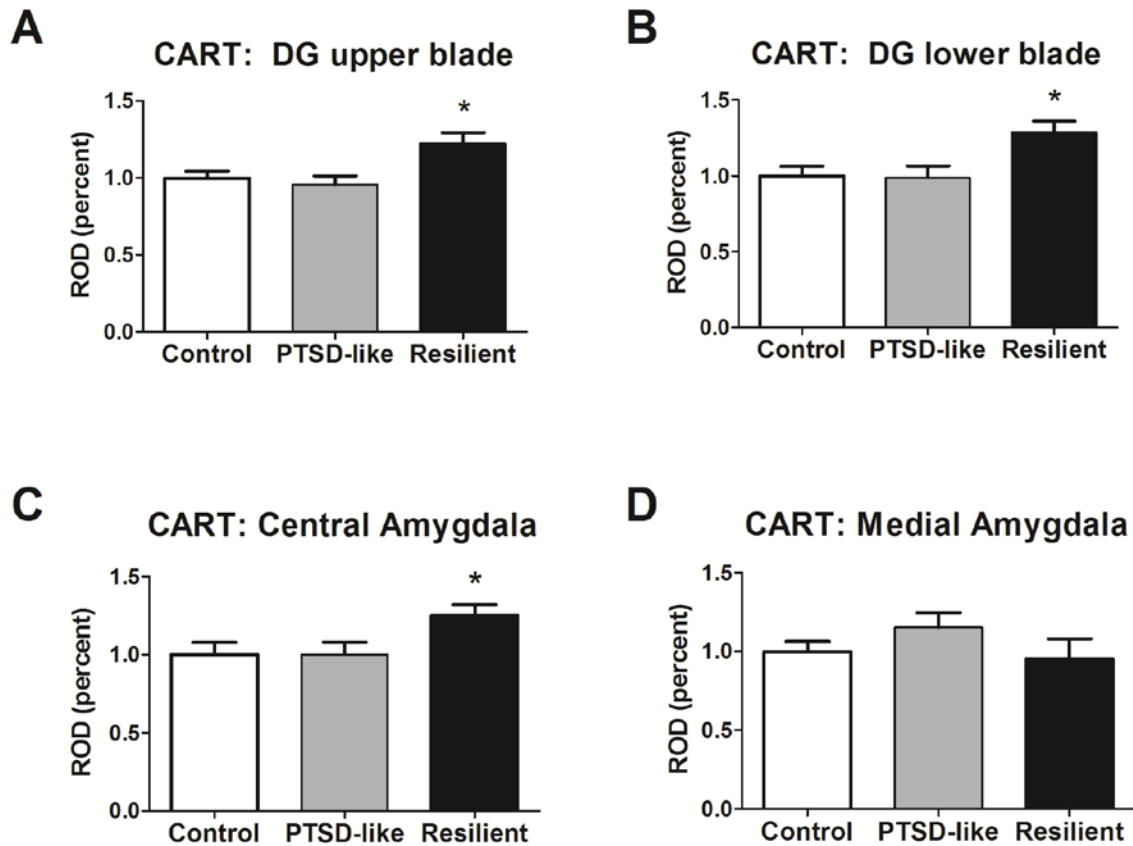


Figure 17. Higher CART mRNA expression levels were found in the hippocampus and central nucleus of the amygdala in stress-resilient individuals. Animals in the Resilient group had higher CART mRNA expression in (A) the top and (B) the bottom blade of the dentate gyrus and (C) the central nucleus of the amygdala ($p < 0.05$). No difference between groups was found in the medial nucleus of the amygdala. Significance is illustrated; *, where $p < 0.05$.

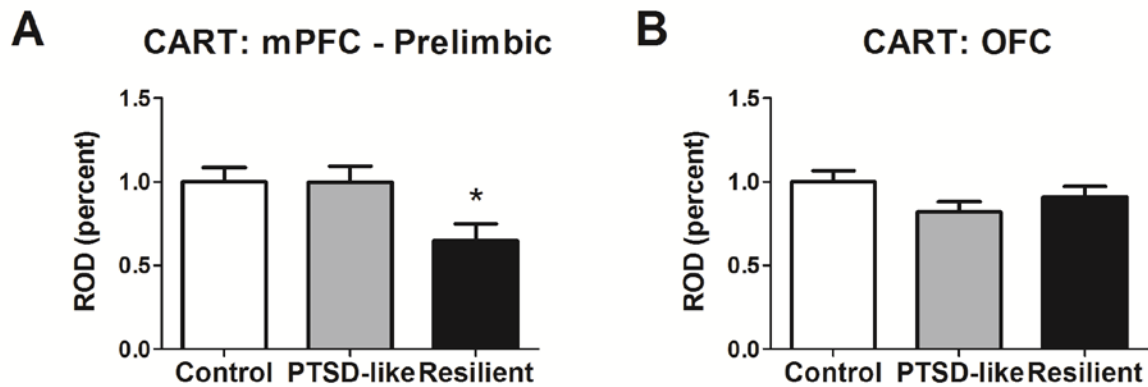


Figure 18. Lower CART mRNA expression levels were found in the prelimbic region of the mPFC in stress-resilient individuals. Animals in the Resilient group had lower CART mRNA expression in (A) the prelimbic region of the mPFC than those in the PTSD-like group ($p < 0.05$). No difference between was found in (B) the orbitofrontal cortex. Significance is illustrated; *, where $p < 0.05$.

CART peptide expression analysis

Densitometry for CART peptide expression was measured on matched sections in the hippocampal formation, amygdala and prefrontal cortex. Nickel-DAB immunolabeling was present in both cell bodies and fiber tracts. Layers of the dentate gyrus that primarily contained immunopositive cell bodies were analyzed separately from layers that contained primarily fiber tracts. Sub-regions including, the granule cell layer (GCL), Subgranular Zone (SGZ), and hilus of the dentate gyrus (DG) and the *Cornu Ammonis* region 3 (CA3) were sampled as shown in Figure 19A and B. . Nuclei of the amygdala, including the central (CE), medial (ME), lateral (LA), basolateral (BLA) nuclei along with the bed nucleus stria terminalis within the amygdalar region (BNST) were identified based on atlas landmarks and sampled separately for densitometry analysis as shown in Figure 19C. In the prefrontal cortex, the cell bodies of the orbitofrontal region were sampled as shown in Figure 19D and layer II/III of the prelimbic (PL) and infralimbic (IL) region of the medial prefrontal cortex (mPFC) were examined as shown in Figure 19E.

In the DG, the upper and lower blades were analyzed separately. A one-way ANOVA showed a group difference in the GCL, in both the upper (Figure 20A) and lower (Figure 20B) blades, $F(2,12) = 3.99$, $p = 0.047$, and $F(2,12) = 4.30$, $p = 0.0390$, respectively. Post-hoc comparisons revealed higher CART expression in the Resilient group as compared to unstressed controls ($p < 0.05$ for both analyses, Bonferroni corrected). An analysis of the SGZ found similar effects. There were group effects in the SGZ adjacent to both the upper (Figure 20C) and lower (Figure 20D) blades of DG, $F(2,12) = 4.68$, $p = 0.0314$, and $F(2,12) = 7.15$, $p = 0.009$,

respectively. Post-hoc analyses revealed that there was higher expression in the Resilient group compared to controls in the upper blade and higher expression than both controls and PTSD-like individuals in the lower blade ($p < 0.05$, Bonferroni corrected). An analysis of the hilus (Figure 20E) also showed a significant difference between groups, $F(2,12) = 5.64$, $p = 0.0187$, and the same effects in the post-hoc comparisons ($p < 0.05$, Bonferroni corrected). In the *cornu Ammonis* region of the hippocampus, clear labeling of fiber tracts was seen in CA3. A one-way ANOVA of the densitometry calculations revealed a trend towards a group effect in CA3a (Figure 20F), $F(2,12) = 3.46$, $p = 0.0653$. However there was a difference between groups in CA3b (Figure 20G), $F(2,12) = 5.37$, $p = 0.0216$. Post-hoc analysis again found increased expression in the Resilient group as compared to unstressed controls ($p < 0.05$, Bonferroni corrected). No differences were found in region CA3c (Figure 20H, $p > 0.1$).

An analysis of CART protein expression revealed contrasting effects in the amygdala. A one-way ANOVA of CE found no differences between groups (Figure 21A, $p > 0.05$). However an analysis of ME (Figure 21B) found a significant group effect, $F(2,14) = 4.37$, $p = 0.0334$. Post-hoc comparisons revealed lower CART expression in the Resilient group as compared to unstressed controls ($p < 0.05$, Bonferroni corrected). Similar results were found for LA (Figure 21C) and BLA (Figure 21D), $F(2,14) = 4.05$, $p = 0.0409$, and $F(2,14) = 4.02$, $p = 0.0418$, respectively. Post-hoc comparisons also found lower expression of CART protein in the Resilient group as compared to controls ($p < 0.05$, Bonferroni corrected). In the intraamygdaloid region of the bed nucleus stria terminalis, (BSTIA, Figure 21E) a

one-way ANOVA also showed differences between groups, $F(2,14) = 6.34$, $p = 0.011$. Resilient also displayed significantly lower CART expression than controls in this region ($p < 0.05$, Bonferroni corrected).

Immunolabeling for CART antibody was very light in the mPFC, as there very few CART-positive cell bodies or fiber tracts. In the prefrontal cortex, CART protein expression in both the prelimbic region and infralimbic region of the mPFC (Figure 22A and B) was analyzed along with the orbitofrontal cortex (OFC Figure 22C). No difference between groups was found in the PL region nor OFC ($p > 0.05$ for all analyses). However there was a significant effect in IL, $F(2,13)=6.61$, $p = 0.0103$. Post-hoc analysis found that there was a reduction in CART expression only in the PTSD-like group ($p < 0.05$, Bonferroni corrected).

NPY and Y5 Receptor mRNA analysis

Densitometry for NPY mRNA expression was conducted in the dentate gyrus, upper and lower blade, and CA2, CA3a, CA3b, CA3c as well as the infralimbic and prelimbic regions of the prefrontal cortex. A one-way ANOVA was conducted between PTSD-like, Resilient and control groups for all regions listed. No differences between groups were found in any region examined ($p > 0.05$ for all groups, data not shown).

Densitometry for Y5 receptor mRNA expression was conducted in the dentate gyrus, upper and lower blade, and CA1, CA2, CA3a, CA3b, CA3c as well as the infralimbic and prelimbic regions of the prefrontal cortex. A one-way ANOVA was conducted between PTSD-like, Resilient and control groups for all regions

listed. No differences between groups were found in any region examined ($p > 0.05$ for all groups, data not shown).

Figure 19. CART peptide expression in the hippocampal formation, amygdala and prefrontal cortex. Photomicrographs of nickel-DAB immunolabeled sections using an antibody against CART neuropeptide display regions sampled for protein expression densitometry and analysis. Sampled regions of interest are labeled within the (A) hippocampal formation, including the CA3a, CA3b, and CA3c (B) the dentate gyrus in enlarged to show detail of layers of the dentate gyrus including the granule cell layer (GCL), subgranular zone (SGZ), and hilus (HIL). (C) Nuclei of the amygdala were sampled from the following regions, central (CE), medial (ME), lateral (LA), Basolateral (BLA), and the intraamygdaloid region of the bed nucleus stria terminalis (BNST). (D) In the prefrontal cortex, an anterior section was analyzed along layer II cell bodies in the orbitofrontal region (OFC). (E) Further posterior, layer II/III of the prelimbic (PL) and infralimbic (IL) regions of the medial prefrontal cortex were sampled.

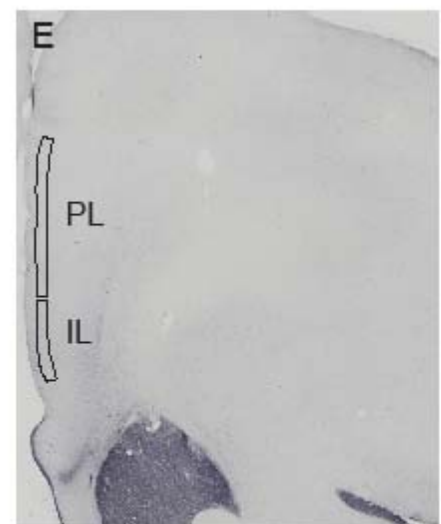
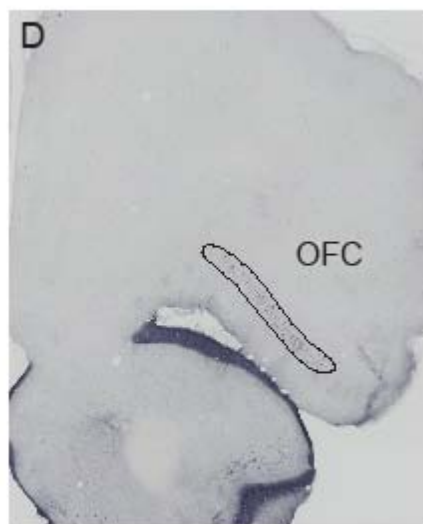
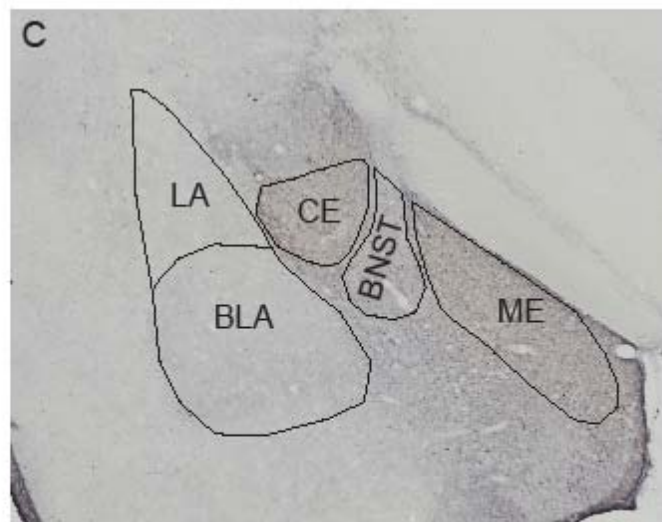
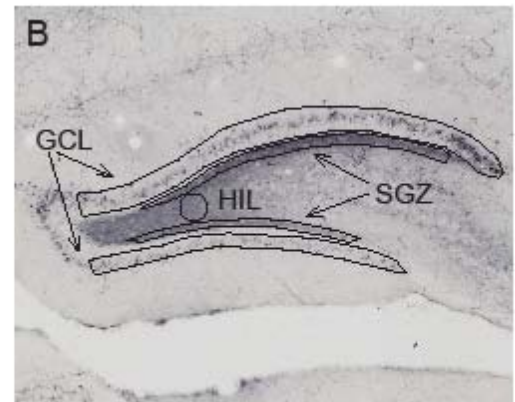
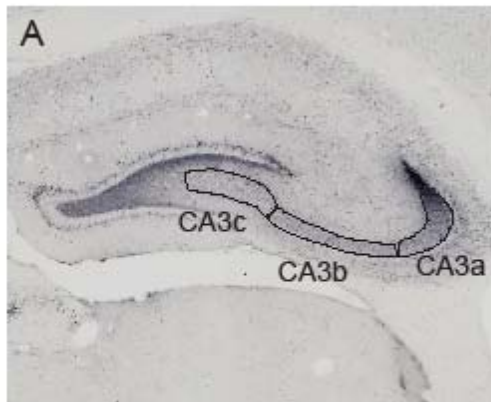


Figure 20. Higher CART protein expression levels were found in the hippocampus in stress-resilient individuals. Animals in the Resilient group had higher CART protein expression as compared to unstressed controls in the granule cell layer (GCL) of (A) the top and (B) the bottom blade of the dentate gyrus, as well as in the regions of the subgranular zone adjacent to (C) the top and (D) the bottom blade of the dentate gyrus. There was also higher CART expression in the Resilient group as compared to the PTSD-like group in SGZ adjacent to the lower blade of the dentate gyrus. ($p < 0.05$ for all comparisons, Bonferroni corrected). Higher CART expression in the Resilient group as compared to controls was also found in (E) the hilus and (G) region CA3b of the hippocampal formation. No significant differences were found between groups in (F) CA3a or (H) CA3c. Significance is illustrated; *, where $p < 0.05$.

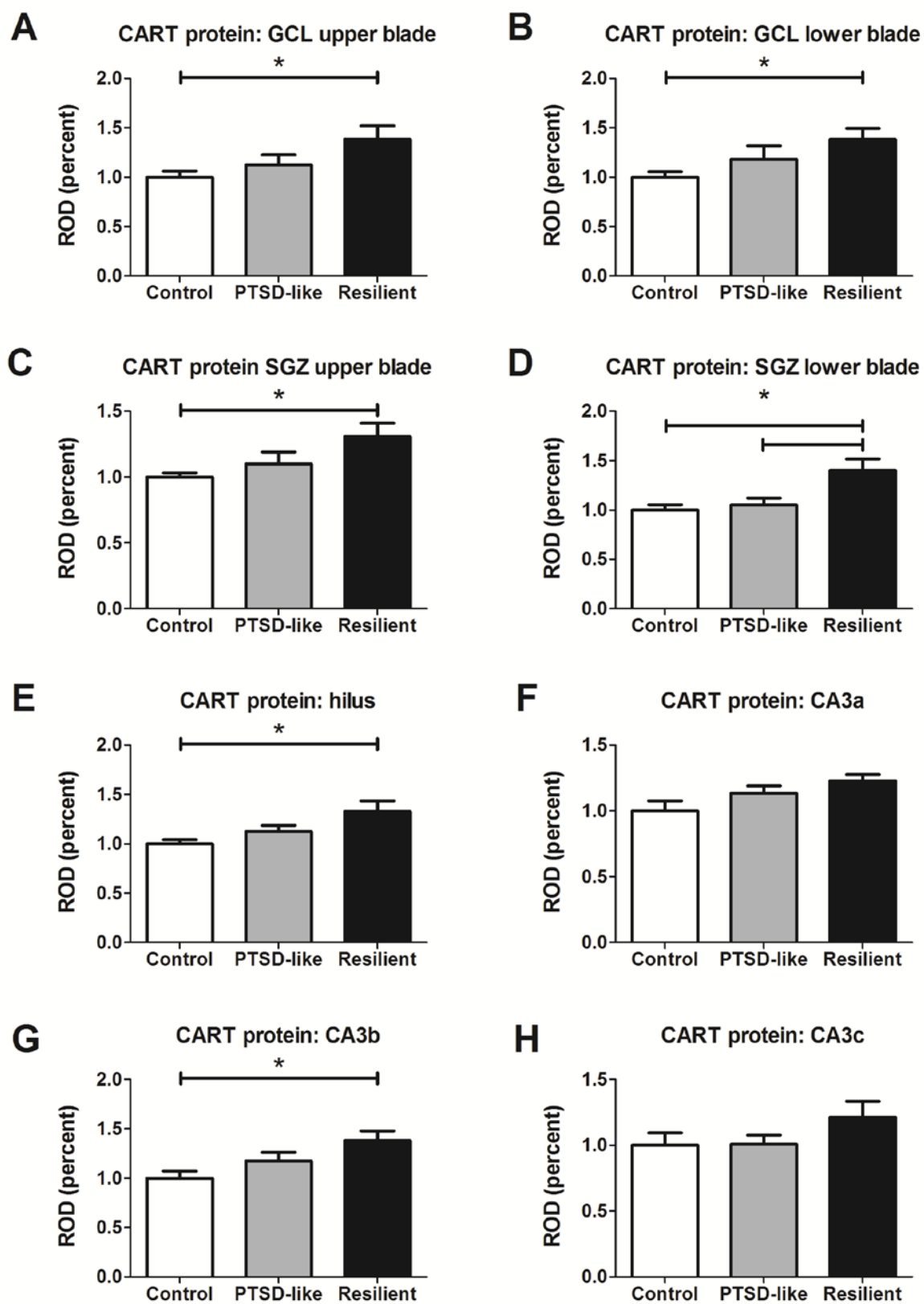
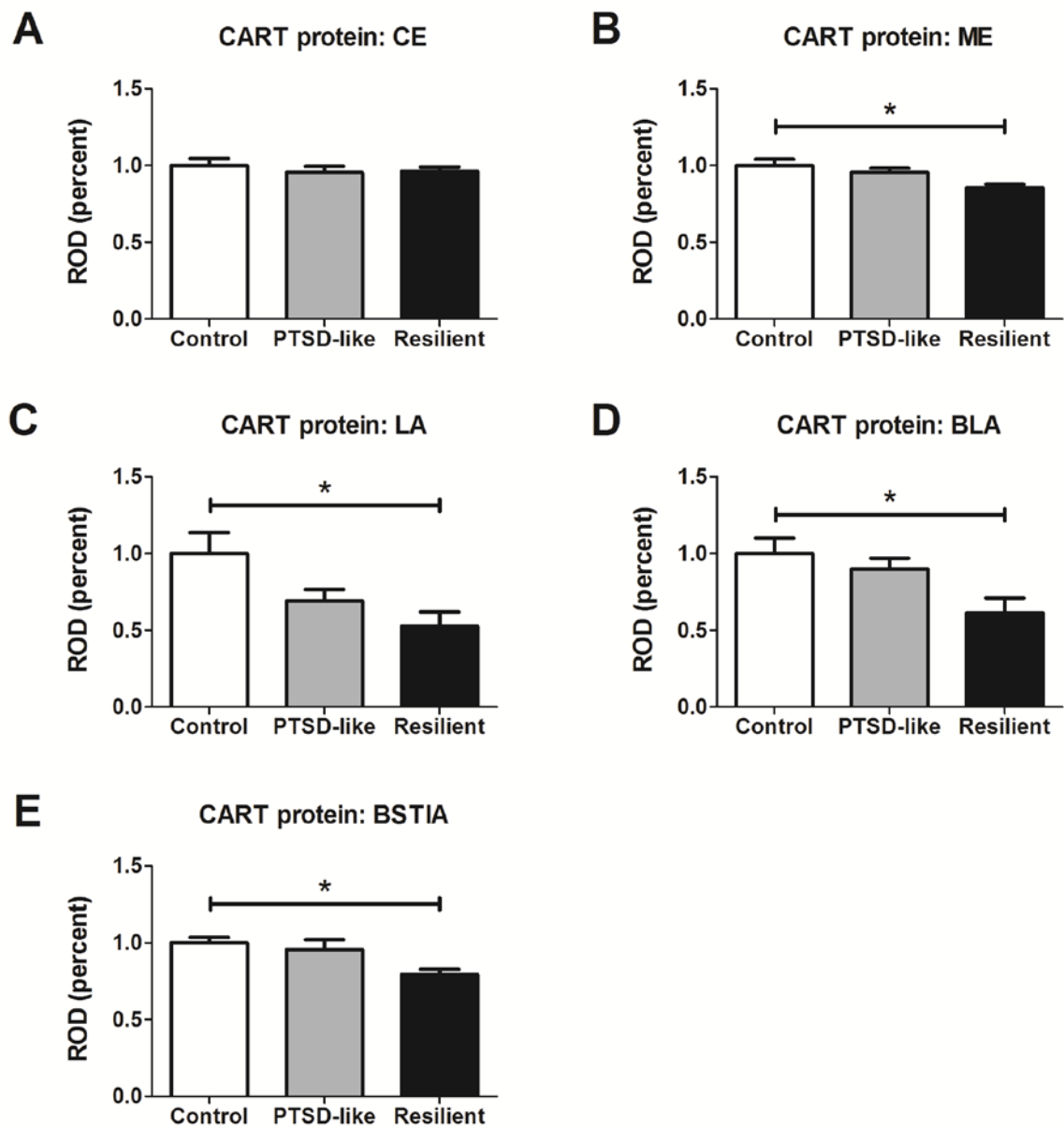


Figure 21. Lower CART protein expression levels were found in the nuclei of the amygdala in stress-resilient individuals. While no differences were found between groups in (A) the central nucleus of the amygdala (CE), animals in the Resilient group had lower CART protein expression as compared to unstressed controls in (B) the medial nucleus of the amygdala (ME); as well as in (C) the lateral nucleus (LA) and (D) basolateral nucleus of the amygdala ($p < 0.05$ for all comparisons, Bonferroni corrected). Lower CART expression in the Resilient group as compared to controls was also found in (E) the bed nucleus stria terminalis, intraamygdaloid region (BNSTIA, $p < 0.05$, Bonferroni corrected). Significance is illustrated; *, where $p < 0.05$.



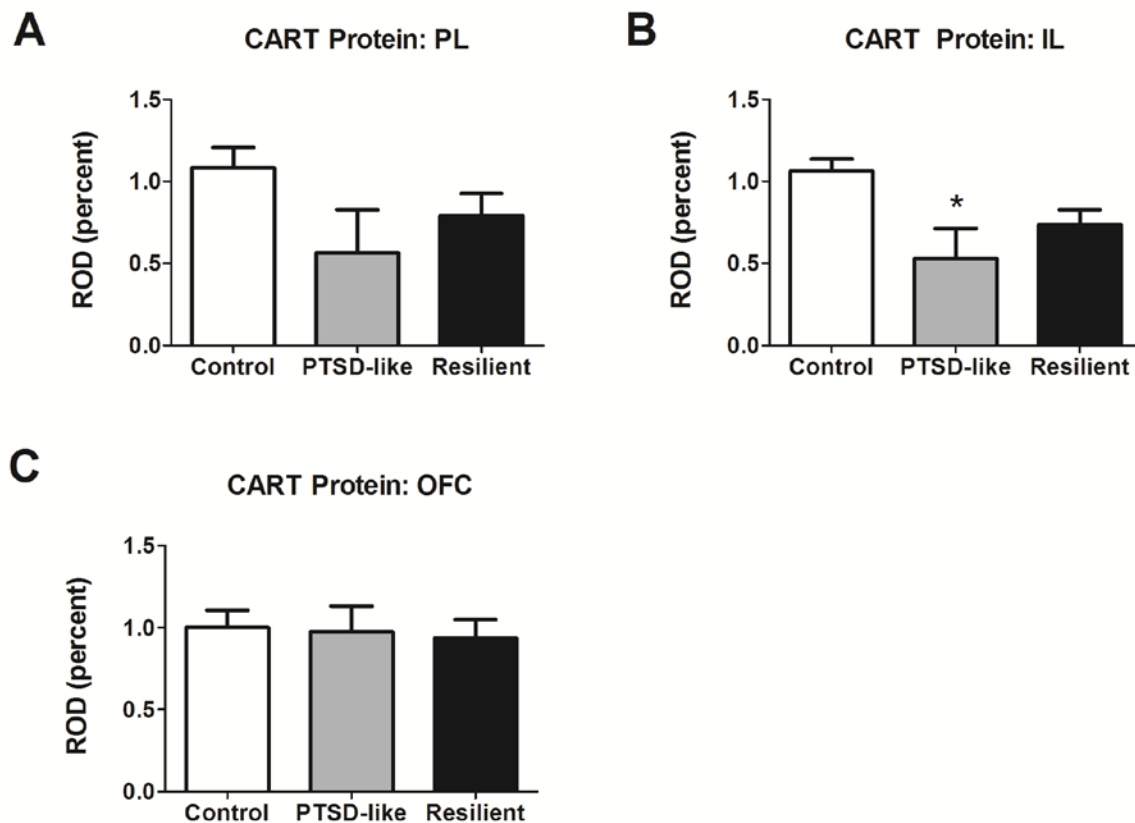


Figure 22. Lower CART protein expression levels were found in the infralimbic region of mPFC in PTSD-like individuals. While no differences were found between groups in (A) the prelimbic region (PL) of the mPFC, individuals in the PTSD-like group had lower CART protein expression as compared to unstressed controls in (B) the infralimbic region (IL) of the mPFC ($p < 0.05$, Bonferroni corrected). (C) No differences between groups were found in the orbitofrontal cortex (OFC). Significance is illustrated; *, where $p < 0.05$.

Discussion

The results of this chapter validate our multimodal stress paradigm as an animal model of PTSD by showing that there is an overall increase in anxiety in stressed animals compared to controls. Additionally, we find individuals that develop anxiety as a result of the stressor (the PTSD-like) and also individuals that appear behaviorally resistant to the effects of stress (the Resilient). Using the criteria set above to separate the PTSD-like from the Resilient, we revealed differences in CART mRNA and protein expression in resilient individuals, a pattern that is in many ways similar to CART mRNA expression in the unstressed low-anxiety individuals described in Chapter 4.

The multimodal stress episode produced a large plasma corticosterone (CORT) increase from baseline levels in both Lewis and F344 rat strains. In fact, rats of both strains produced elevated CORT levels immediately after a 10-minute psychogenic cat odor exposure that were almost as high as the response to immobilization and tail bleed. However, it was unexpected that the Lewis and F344 lines would produce nearly identical CORT responses. Historically, Lewis rats are thought to have basal CORT levels similar to those of other outbred strains, such as the Sprague-Dawley, but when challenged by a stressor, they produce a hypoactive response in all measures of the Hypothalamic-Pituitary-Adrenal (HPA) axis. F344 rats on the other hand, are sometimes, although not always, found to have higher basal CORT levels and a much larger CORT response to stress (Griffin et al. 1991; Dhabhar et al. 1993; Gomez et al. 1996; Cohen et al. 2004; Cohen et al. 2006). The plasma CORT levels reported in this study are both particularly high in the

Lewis strain (typical range 100-200 ng/ml after stress) and particularly low for the F344 strain (typical range 400-600 ng/ml). This poses an issue with the purity of the genetic line of the rats produced by the supplier. Yet, there were still significant differences between the strains. Lewis rats from Charles River Labs (Wilmington, MA) consistently weigh less than Sprague Dawley rats (data not shown). Further, the anxiety behavior of the Lewis rats was consistently higher than both SD and F344 strains; control and stressed Lewis rats spend less time in the open arms of the EPM.

Yet the even the anxiety behavior produced was not in the same range as the published results that this experiment was modeled after. Cohen and colleagues found that unstressed Lewis rats spent about 4% of the time in the open arms of the EPM, which reduced to an average of about 1.4% with stress, and that F344 rats spent an average of 60% of their time in the open arms which reduced down to about 10% with stress (Cohen et al. 2006). Additionally, they found that about 90% of stressed Lewis rats exhibited extreme behavioral responses, meaning they never entered the open arms of the EPM (Cohen et al. 2004). In the above multimodal stress experiments, only about 10% of the stressed animals produced these extreme behavioral responses. Pilot studies using only a 10-minute cat odor exposure (as per Cohen et al., 2004) were even less effective at eliciting a behavioral response (see Chapter 3). It was for this reason that the multimodal stressor was chosen, in order to enhance the effects of the cat odor stress, but this paradigm still allows for a significant percent of the animals to remain resilient to the stressor (20-35%).

There is some evidence that Lewis rats bred in different facilities may vary significantly in their CORT response and behavior (Stohr et al. 1999). It is also possible that the behavioral facility, cat odor stress setup, and EPM conditions differ so greatly between laboratories that we find both a blunted response in the F344 strain in both stress-induced CORT levels and in EPM behavior, and an enhanced response of both CORT and behavior in the Lewis strain; but this seems unlikely. The evidence leans towards a conclusion that there is a dilution of the inbred strains causing their hormone and behavioral responses to move towards the outbred SD profile.

Despite these problems, the multimodal stress was able to induce significant changes in anxiety behavior in the Lewis strain. Overall stressed rats spent less time in the open arms of the EPM and had a longer latency to first enter the open arms. Unfortunately, it appeared that the bird song audio cue did not enhance anxiety when set as a conditioned stimulus for the traumatic experience and played as a reminder cue in the EPM. Several different scenarios could be happening to prevent cue –induced enhancement of anxiety. One suggestion would be that the bird song was not strongly associated with the traumatic experience or was not an effective reminder cue for the traumatic experience. There is evidence against this possibility in that animals in the song cued groups had a higher CORT response after hearing the song than animals that either heard the song for the first time that day or did not hear the song at all that day. This would suggest that the cue was sufficient to induce an autonomic conditioned response but was not effective at reducing the time spent in the open arms of the EPM.

While the song itself did not increase CORT levels when played in the EPM (as shown in the no song/song groups), regardless of stress, there was a main effect of song cue on anxiety behavior in those animals that heard the song on both stress/control exposure day and in the EPM. This would suggest that the song may have been consolidated as a slightly arousing stimulus for the control group, although this did not affect their EPM behavior. Another possible reason that the song cue was not effective in enhancing anxiety behavior is that the EPM may not be an ideal environment to conduct a test of the recollection of a traumatic experience, or possibly the EPM measures are not sensitive enough to show a small enhancement of fear behavior which may look different than the types of anxiety behavior measured by this maze.

Even without the use of a cue to serve as reminder of the stressful experience, there was an overall effect of stress on anxiety behavior. The correlation between control open field and EPM behavior was lost in stressed animals, demonstrating that anxiety profiles may have changed after stress. However, from the graph (figure 16B), it was clear that there was a lot of variability; there were individuals whose behavior had remained the same before and after stress and also those that had changed. From the previous work in Chapter 4, we had also recognized that baseline anxiety levels could influence the dendritic morphology and molecular profile of the brain; therefore, we wanted to select for those animals that truly changed as a result of the multimodal stress experience. Equally as interesting were the animals that didn't change after a stressful

experience, as these may provide insight into alterations in molecular profiles that produce a resilient individual in the face of stress.

CART mRNA expression and peptide were analyzed in individuals that present PTSD-like or resilient anxiety profiles after stress. While a plausible prediction would have been that since the PTSD-like group contained the individuals that had changed in their baseline stress levels we should find differences in their molecular profile, this was not the case. In nearly all regions examined, it was the resilient group that showed a change in expression, while the PTSD-like group tended to look more like controls. CART mRNA expression increased in resilient animals in the dentate gyrus of the hippocampal formation, central nucleus of the amygdala, and decreased in the prelimbic region of the prefrontal cortex. These results appeared consistent to what was found in the anxiety profiles of unstressed controls. The calmer animals showed higher CART expression in the dentate gyrus and a correlation of lower expression with lower anxiety in the prefrontal cortex. However, the resilient profile differed from the calm profile in the amygdala, where increased CART expression correlated with high anxiety. The amygdala is highly involved in the fear response and the formation of fear memories, and it is possible that it serves a different purpose in response to stress. There is some evidence that the projections coming from the central nucleus of the amygdala that are involved in encoding a stressful experience are different than the ones that are involved in encoding for basal anxiety levels (Van Bockstaele et al. 1999). While the role of CART in these two amygdala pathways are not clear,

in may be the combination of activity in these pathways that determines total CART mRNA expression.

CART protein followed a similar profile to the mRNA expression with a few exceptions. CART antibody allowed for better resolution, which allowed for visualization of immunolabeled cell bodies as well as areas and nuclei that contained a high concentration of CART-positive fiber tracts. For this reason, the hippocampal formation was divided into several more regions to differentiate between areas containing primarily cell bodies (such as GCL) compared to those with mostly fiber tracts (SGZ, hilus, CA3). This enhancement of resolution allowed us to also analyze immunolabeled fiber tracts in many nuclei of the amygdala that we were unable to visualize with mRNA expression, including the LA, BLA and BSTIA.

Similar to the mRNA profile, CART protein expression generally increased with resilience in the DG in most areas examined. CART positive cell bodies in the GCL are most likely part of the mossy fiber pathway (Seress et al. 2004; Abraham et al. 2007), and may make up the bulk of the CART positive fiber tracts projecting into the hilus and into CA3, both regions where we also see increases in expression with resilience. In the amygdala, the effects were opposite; CART protein expression was generally reduced in resilient individuals, particularly in areas that contained mostly immunolabeled fiber tracts. This results is supported in the literature, as CART peptide injected into the amygdala has been shown to be anxiogenic on several behavioral measures (Dandekar et al. 2008; 2009), and CART mRNA expression has been found to increase with stress in the CE but not

ME (Hunter et al. 2007). The central amygdala appears to be an interesting area, as we see varying effects in this region depending on whether we measure mRNA or protein, and whether we are looking at behavior after stress or just basal anxiety. While all other amygdala nuclei examined show a reduction in CART protein expression with stress, only becoming significant in the behaviorally resilient animals, CART levels in CE do not appear to change. While many explanations are possible, the connectivity between the other nuclei of the amygdala and CE are quite different. While LA/BLA, and ME receive and integrate input from lower order and higher order sensory processing areas, CE mainly receives both excitatory and inhibitory input from the other nuclei of the amygdala as well as inhibitory input from higher cortical areas, and is the main region of output of the amygdala response. More research is necessary to better understand the role of CE in the CART neuropeptide system, which is considered further in the main discussion.

The IL region of the mPFC was also unique in that this was the only region in the entire study where we found significant differences in the PTSD-like animals. While there were trends towards a reduction in CART expression in the Resilient individuals, this was not significant. It appeared that, in this case, resilient animals were the ones that were resisting change in CART expression. However, this was another instance where the protein levels did not follow the mRNA expression trends. As the IL region of mPFC is known to inhibit amygdala activity, and necessary for extinction learning (Quirk et al. 2003; McDougall et al. 2004), it is possible that increased CART protein is indicative of active inhibitory neurons in this region, a sign of proper inhibitory tone.

This result introduces another important factor; although we only found significant expression changes in the resilient animals, there was a trend for the PTSD-like animals to change from baseline in the same direction (and a trend for Resilient animals to change from baseline, in the case of the IL region of mPFC), particularly in protein expression. This supports the idea that resilience is an active process, and those animals that produce a sufficient molecular change in response to a stressor are actually the ones that retain their pre-stress behavior, while those that cannot mount the appropriate molecule response above a certain threshold are destined to become more anxious.

The question remains, are these changes in CART necessary for the resilience profile and retention of pre-stress anxiety levels, or are they just a secondary effect? To answer this, better understanding of what is happening upstream of the CART changes is necessary. One possible candidate molecule working upstream of CART would be NPY. Increased NPY levels in both blood and brain appear to be anxiolytic (Thorsell et al. 1998; Heilig 2004) and positively influence stress resilience in both animals and humans (Morgan et al. 2000; Morgan et al. 2002; Yehuda et al. 2006). NPY-positive axon terminals have been shown to be apposed to dendrites in CART positive neurons in the hypothalamus and medial nucleus of the amygdala (Broberger 1999). While NPY may be acting on CART expression, no differences were found in NPY mRNA expression levels between stressed animals with PTSD-like and resilient behavior or in unstressed controls with different anxiety profiles in the above studies. Other candidate molecular systems are discussed and tested in the following chapter.

Chapter 6: Mechanisms for stress resilience and prevention of PTSD

Based on the results of Chapter 5, the multimodal stressor proved to be effective for eliciting a long-term anxiety response in the Lewis rats. This paradigm was utilized as a model of PTSD in the following experiments. The neurotransmitter glutamate and the NMDA family of receptors have been implicated in the encoding of fear memories and in the enhancement of anxiety after stress (Maren et al. 1996; Adamec et al. 1998; Blair et al. 2001). Here we administer the NMDA receptor antagonist CGP43487 prior to the multimodal stress exposure to test whether NMDA receptor blockade during stress can prevent stress-induced changes in behavior. The stress hormone Corticosterone (CORT) and its receptors also play a large role in eliciting anxiety behavior after stress (McEwen et al. 1988; Raber 1998; Calvo et al. 2001; Mitra et al. 2008). However, the Lewis rat strain has been shown to have a blunted CORT response immediately following stress, and this has been implicated in the PTSD-like symptoms found in this strain (Cohen et al. 2006). As administration of additional CORT during the stress episode has been shown to eliminate the PTSD-like effects on behavior, we attempted to replicate this finding using a non-invasive route of CORT administration. Neuropeptide Y (NPY) expression has been strongly associated with reduced anxiety and behavioral resilience to stress (Heilig et al. 1993; Yehuda et al. 2006; Gutman et al. 2008; Sajdyk et al. 2008), however, some of the NPY receptors have opposing effects on anxiety behavior (Thorsell et al. 2002). Here we used a drug to block the NPY Y5

receptor during stress. As the Y5 receptor antagonist, Lu AA33810, has only been shown to be effective with chronic treatment (Walker et al. 2009), a chronic stressor was utilized to examine the ability of this drug for enhancing stress resilience.

Experimental Paradigm

Experiment 1: NMDA-Receptor blockade during an acute multimodal

stress exposure. Adult male Lewis rats were divided into 6 groups, run in two cohorts of 9 animals per group: control/no injection, control/vehicle, control/NMDAR Ant, stress/no injection, stress/vehicle, stress NMDAR Ant. Animals in the injection groups received an s.c. injection with 5mg/kg of the NMDAR antagonist CGP43487 or vehicle. In both studies, a five-minute open field test occurred several days prior injections and/or stress or control exposures. Seven days after stress or control exposure, rats were placed in the elevated-plus maze for five minutes. Brains were collected via rapid decapitation or perfusion on the same day.

Experiment 2: Noninvasive corticosterone treatment during an acute

multimodal stress exposure. This experiment was conducted in two cohorts (first cohort for plasma analysis, 6 per group; second cohort for behavior, 12 per group). In both studies, a five-minute open field test occurred several days prior to CORT treatment and stress exposure in order to divide the animals evenly into groups based on anxiety measures; control/vehicle, control/CORT, stress/vehicle, stress/CORT. CORT treatment consisted of changing the regular water bottles for

water containing 400µg/ml corticosterone and 2.4% ethanol, while controls receive bottles with 2.4% ethanol, for 12 hours prior to stress/control exposure.

In the first cohort, animals were sacrificed by rapid decapitation immediately after the stress or control exposure (40 minutes after onset of exposure). Blood was collected and processed for hormone analysis and the brains flash frozen. For the second cohort, seven days after stress exposure, rats were placed in the elevated-plus maze for five minutes. Brains were collected via rapid decapitation less than two hours after maze exposure, flash frozen on dry ice, and processed for in situ hybridization.

Experiment 3: Y5 receptor antagonist treatment during chronic stress:

Adult male SD rats were divided into four groups: control/normal chow, control/ Y5 Ant, stress/normal chow, stress/ Y5 Ant (12 animals per group, 48 total). Prior to the start of the experiment all animals were run in the open field and divided evenly across groups based on anxiety behavior. Administration of chow containing Y5 Receptor Antagonist Lu AA33810 (30 mg/kg/day) or normal chow began four days before the start of the stress experiment. A sucrose preference was performed immediately after the first and last day of stress for all groups. In cage behavior was monitored for 2 hours after stress day 2 and day 20 for all groups. Animals were weighed once a week. Twenty-four hours after the last stress episode, all animals were placed in the EPM for five minutes.

Animals were sacrificed by rapid decapitation five minutes after removal from the maze, and blood collected for plasma hormone analysis. Brains were removed and

flash frozen for *in situ* hybridization processing. *In situ* hybridization using radiolabeled oligonucleotide probes for NPY and Y5 receptor were conducted on prepared hippocampal and mPFC sections. Matched sections on the resulting autoradiographs were analyzed by densitometry and compared by two-way ANOVA.

Results

Experiment 1

Behavior in the EPM was examined seven days after NMDA receptor antagonist treatment and stress exposure. A two-way analysis of variance found no differences in the percent time spent in the open arms (Figure 23A, $p > 0.1$) nor in the number of open arm entries (Figure 23B). As the pre-stress open field test showed a wide range of anxiety levels prior to dividing the animals into experimental groups, a comparison of anxiety behavior was accomplished by calculating z-scores (calculated as percent of the population) for pre-experiment and post-experiment behavior. The pre-experiment z-score was obtained by taking the individual data for time spent in the center of the open field, and subtracting the mean of the entire population. This number was then divided by the standard deviation of the entire population, and adjusted to a z-score calculated percentile. The post-experiment z-score was obtained by taking the individual data for time spent in the open arms of the EPM, and subtracting the mean for the Control/No Injection group, which should represent an unchanged population. This was then divided by the standard deviation of the same control group, and adjusted to a z-score percentile. In order to

emphasize whether changes from pre-experiment behavior were in the positive (less anxiety) or negative (more anxiety) direction, z-score percentiles for post-experiment behavior were subtracted from pre-experiment behavior to acquire a difference score (Figure 23C). A two-way ANOVA of difference scores by group did not reveal any significant effects ($p > 0.10$ for both analyses), however, these numbers to appear to highlight the changes taking place in the experimental groups.

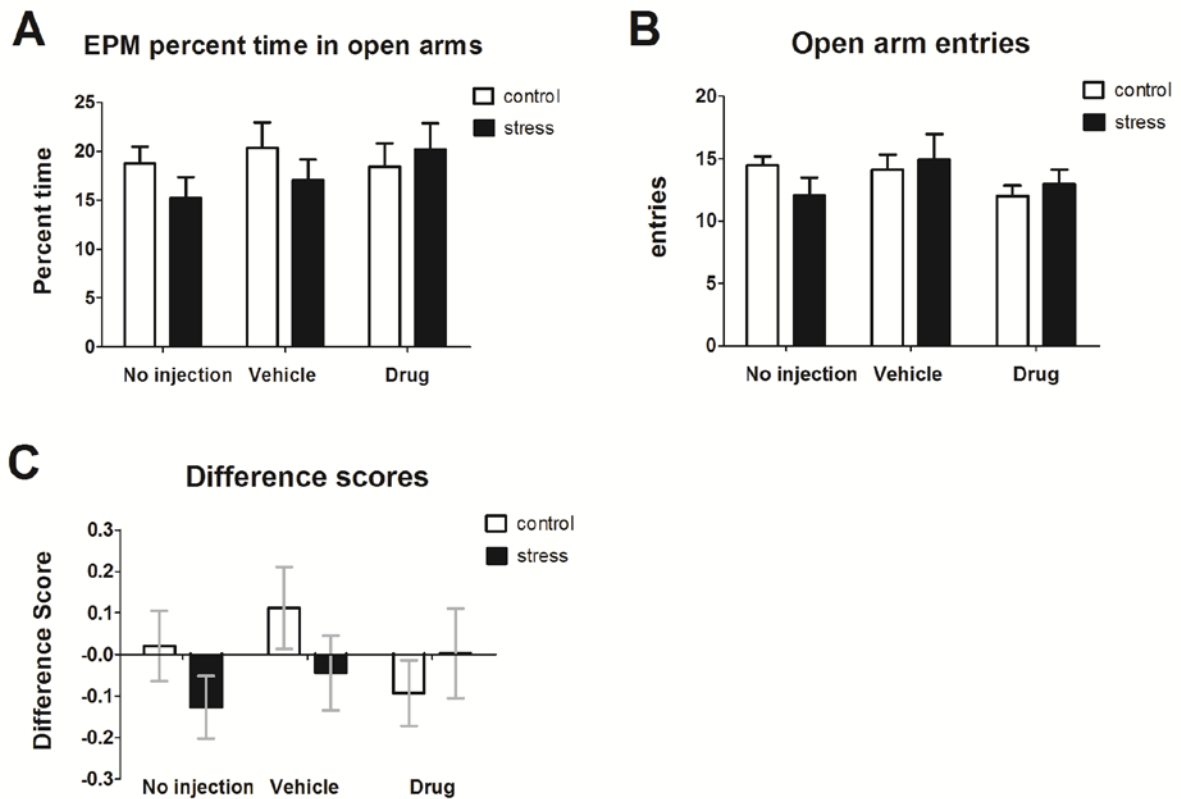


Figure 23. Effects of NMDA receptor antagonist on acute multimodal stress

remain unclear. There is no effect of Stress or NMDA receptor antagonist

treatment on (A) percent time spent in the open arms of the EPM, nor (B) number of open arm entries ($p > 0.05$). Although not significant, difference scores between open field measures and (C) percent time spent in the open arms in the EPM were obtained to show overall trends in behavior for each group.

Experiment 2

Plasma corticosterone (CORT) was analyzed from the blood collected from the first cohort immediately after stress exposure (Figure 24). A two-way ANOVA found a significant main effect of stress, $F(1,19) = 9.60$, $p = 0.0059$, a main effect of CORT treatment, $F(1,19) = 6.75$, $p = 0.0177$, and a stress by CORT treatment interaction $F(1,19) = 11.2$, $p = 0.0034$. Post-hoc comparisons revealed that animals in the stress/No CORT group had a stress-related increase in plasma CORT levels as compared to controls ($p < 0.001$, Bonferroni corrected).

In the second cohort, behavior in the EPM was examined seven days after CORT treatment and stress exposure. A two-way ANOVA found no differences in the percent time spent in the open arms (Figure 25A,) nor in the number of open arm entries (Figure 25B), however, there was a trend towards significance for the stress by CORT treatment interaction $F(1,42) = 3.18$, $p = 0.0819$. As the pre-stress open field test showed a wide range of anxiety levels prior to dividing the animals into groups, a comparison of anxiety behavior was accomplished by calculating z-scores as described above for percent time spent in the open arms of the EPM (Figure 25C) and open arm entries (Figure 25D). In order to emphasize whether changes from pre-experiment behavior were in the positive (less anxiety) or negative (more anxiety) direction, z-score percentiles for EPM behavior were subtracted from open field behavior to acquire a difference score. A two-way ANOVA of difference scores did not reveal any significant effects ($p > 0.10$ for both analyses), however, these numbers to appear to highlight the changes taking place in the experimental groups.

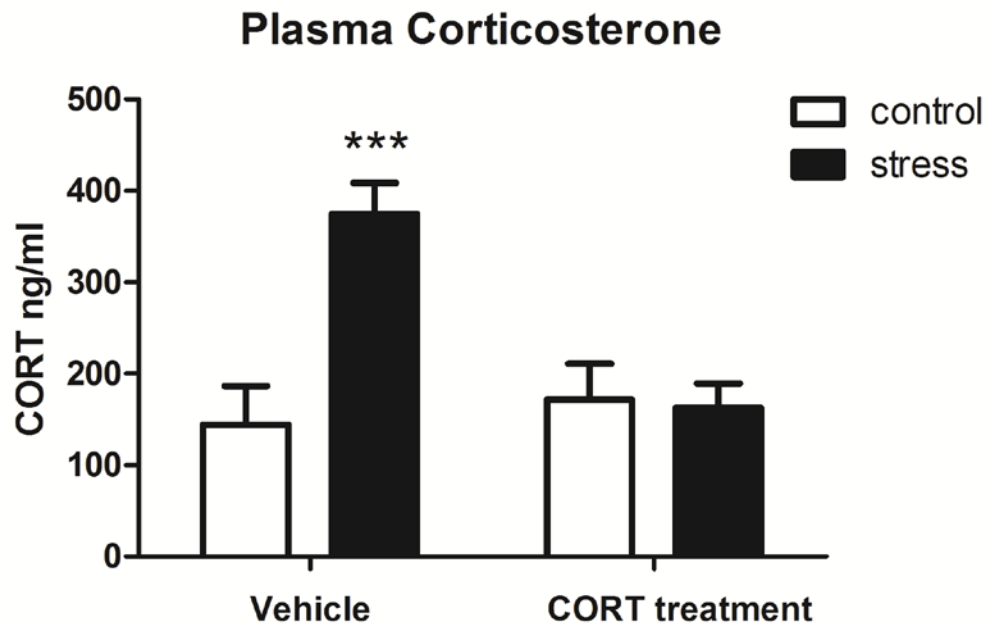


Figure 24. **Post-stress plasma Corticosterone (CORT) is inhibited by 12 hours of CORT treatment.** A two-way ANOVA found a main effect of stress, CORT treatment and a stress by CORT treatment interactions ($p < 0.05$ for all analyses). Post-hoc comparisons showed that stress significantly increased plasma CORT levels in the vehicle treated group. Significance is illustrated; ***, where $p < 0.001$.

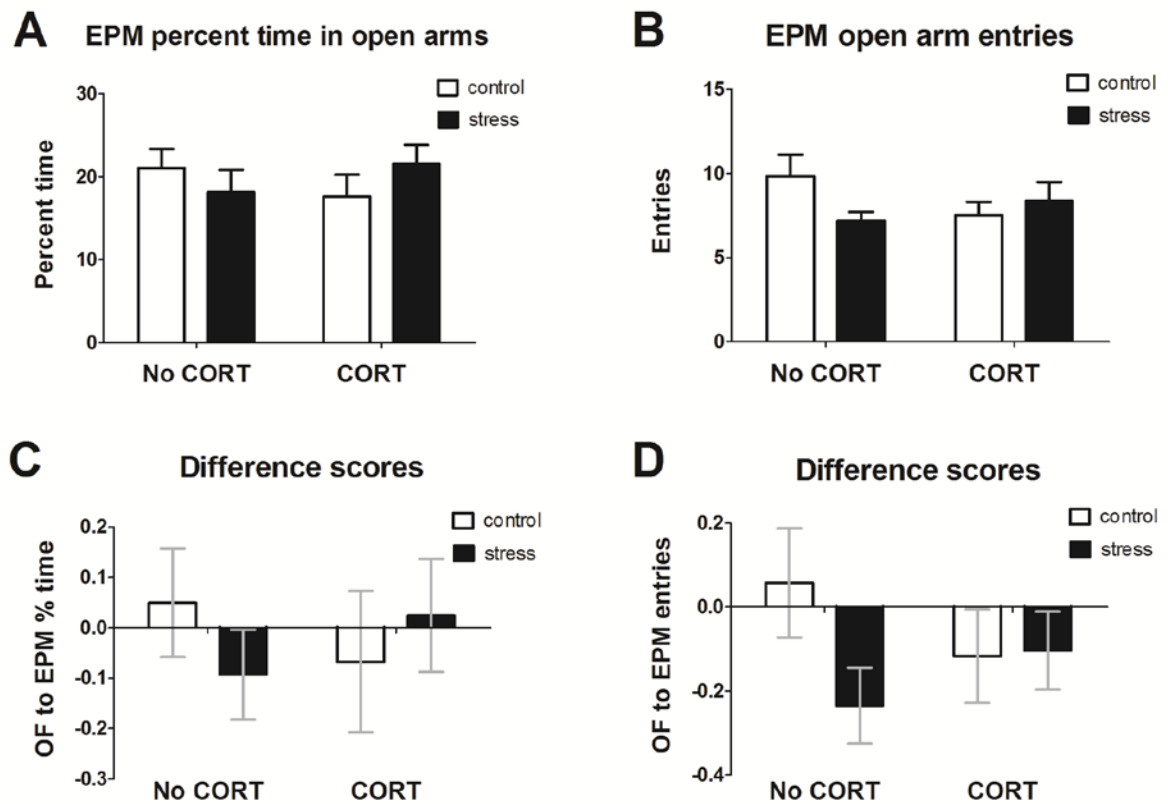


Figure 25. Effects of CORT treatment on the acute multimodal stress appear unclear. There is no effect of Stress or CORT treatment in the drinking water 12 hours prior to stress or control exposure on (A) percent time spent in the open arms of the EPM, nor (B) number of open arm entries ($p > 0.05$). Although not significant, difference scores between open field measures and (C) percent time spent in the open arms, or (D) number of open arm entries in the EPM were obtained to show overall trends in behavior for each group.

Experiment 3

Individual weights were collected before stress and throughout the stress procedure (Figure 26). A repeated measures ANOVA demonstrated an effect of time, $F(5,215) = 1639$, $p < 0.0001$, demonstrating that all animals gained weight over the 6 weeks they remained in the facility. There was also a time by treatment interaction, $F(15,215) = 6.92$, $p > 0.0001$. Two-way ANOVAs comparing weights for each week revealed that there was a main effect of stress on weight during week 5 and week 6 of the experiment ($F(1,44) = 14.7$, $p = 0.0004$, and $F(1,44) = 12.2$, $p = 0.0011$, respectively); however there was no effect of drug treatment or drug by stress interaction ($p > 0.05$).

A two-way ANOVA for sucrose preference found no effect of stress or drug treatment on sucrose preference on Day 2 or Day 20 of the study ($p > 0.05$, Figure 27A and B, respectively). Bonferroni corrected post-hoc analyses did not show significant differences between stress and control in the No Drug group, although there was a trend for the stressed animals to consume less sucrose water on Day 2 ($p = 0.147$), which was significant when the No Drug groups were compared by a two-tailed student's t-test, $t(6) = 2.57$, $p = 0.0425$. This comparison was not significant by Day 20 ($p > 0.1$).

In cage behaviors were counted within the first 2 hours after restraint stress, controls matched for time of day. Aggressive behaviors varied widely across cages (3 rats/cage), from no aggression noted up to 17 aggressive acts in two hours; however, a two-way ANOVA found no differences in behavior either on Day 1 or Day 21 of stress (Figure 27C and D, $p > 0.1$ for all groups). This variability was

found both within groups as well as within cages measured over time; a two-way ANOVA with repeated measures comparing Day 1 and Day 21 found no effect of time, $F(1,12) = 3.15$, $p = 0.101$.

A two-way ANOVA of EPM behaviors measured after chronic stress found no main effects on the percent time spent in the open arms (Figure 28A) nor any significant Bonferroni corrected post-hoc comparisons ($p > 0.05$ for all analyses). There was a main effect of stress on the total number of open arm entries (Figure 28B), $F(1,42) = 4.56$, $P = 0.0386$. Further, there were no significant main effects for the number of times the head reached the end of the open platforms (Figure 28C). Although the Bonferroni corrected post-hoc comparisons for these three measures were not significantly different between Stress and Controls in the No Drug group ($p > 0.05$), we had predicted that we would find a stress-induced increase in anxiety in those animals who did not receive the Y5 antagonist. A two-tailed unpaired student's t-test showed that stressed animals entered the open arms less often, $t(20) = 2.35$, $p = 0.0293$, and there was a trend for the stressed animals to spend less time in the open arms, $t(20) = 1.74$, $p = 0.0973$, and to reach the end of the open platforms less often, $t(20) = 1.762$, $p = 0.0933$.

As the pre-stress open field test showed a wide range of anxiety levels prior to dividing the animals into experimental groups, a comparison of anxiety behavior was accomplished by calculating z-scores as described in the NMDA receptor antagonist study above for the percent time spent in the center of the open field and the percent time spent in the open arms of the EPM. In order to emphasize whether changes from pre-experiment behavior were in the positive (less anxiety) or

negative (more anxiety) direction, z-score percentiles for post-experiment behavior were subtracted from pre-experiment behavior to acquire a difference scores (Figure 28D). A two-way ANOVA of difference scores by group did not reveal any significant effects ($p > 0.10$ for all analyses), however, these numbers to appear to highlight the changes taking place in the experimental groups.

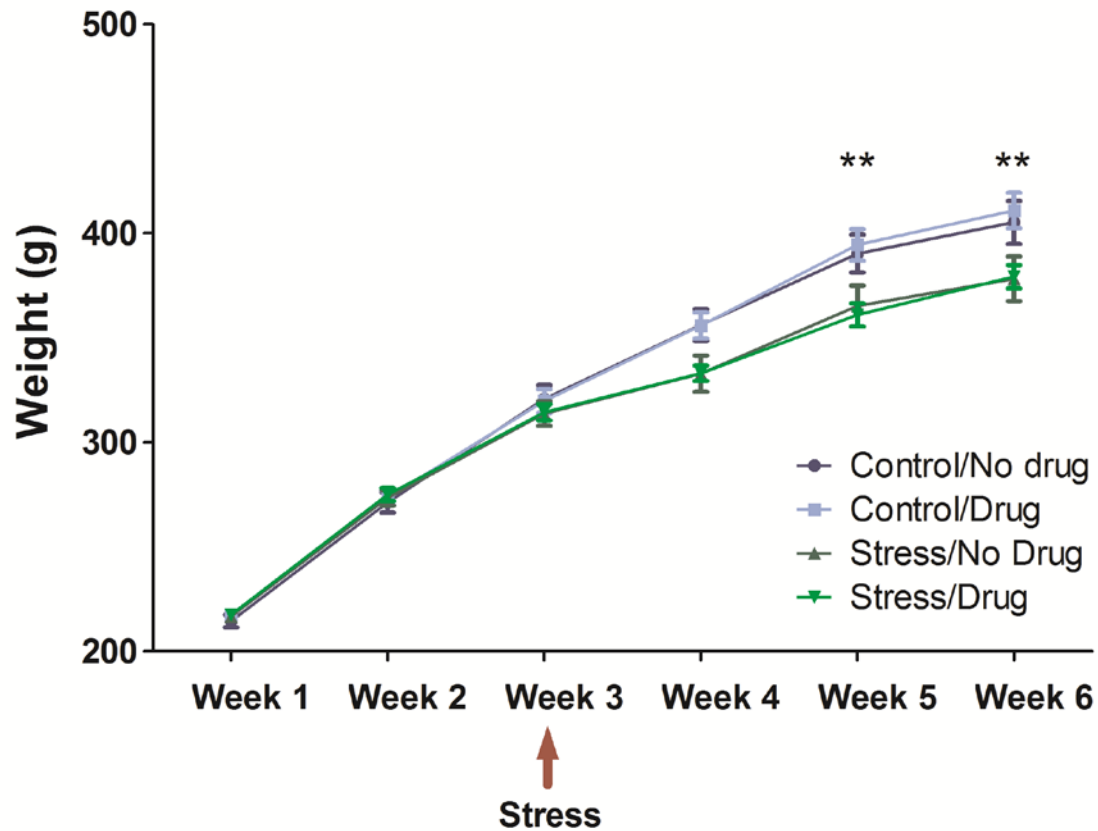


Figure 26. **Chronic restraint stress reduces weight gain regardless of Y5 receptor antagonist treatment. Stress began at the start of week 3, as noted on the figure.** A two-way ANOVA with repeated measures revealed that animals in the stress groups weighed less than controls; drug treatment had no effect on weight gain. Significance is illustrated; **, where $p < 0.01$.

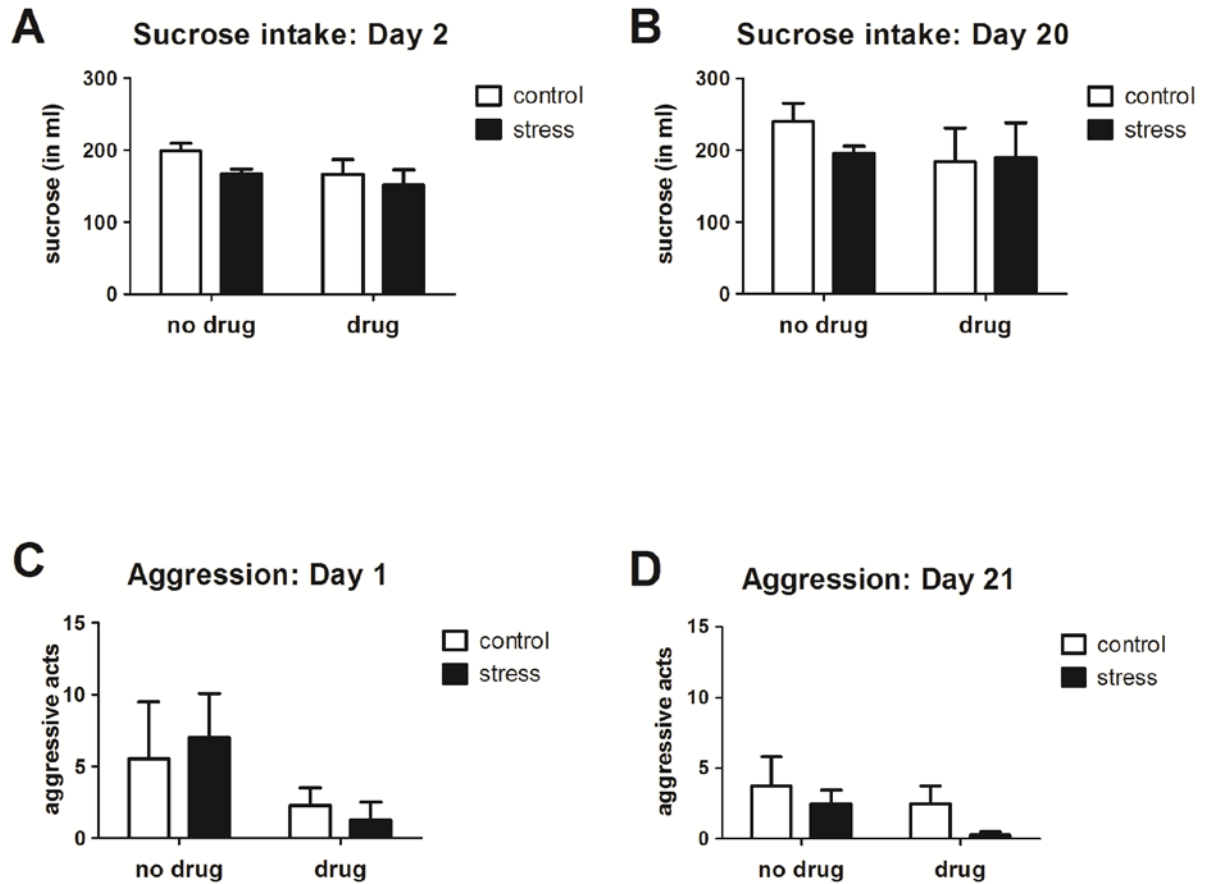
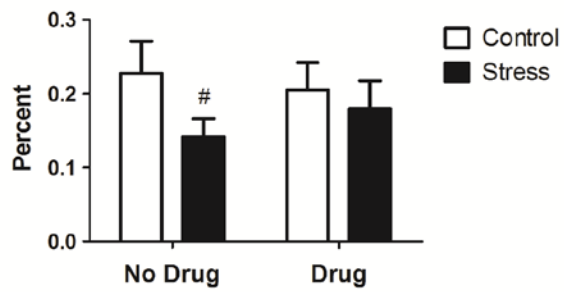


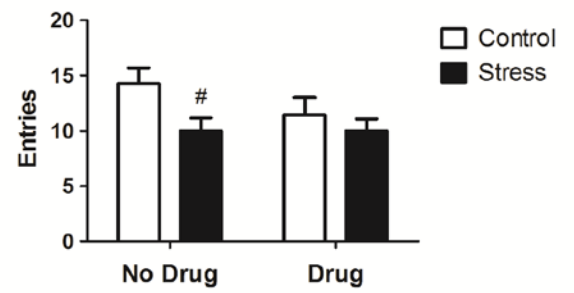
Figure 27. Restraint stress and Y5 receptor antagonist treatment have no effect on sucrose preference or in cage aggression. A two-way ANOVA of behaviors measured at the start and end of the 21-day stress period found no effects of stress or Y5 antagonist drug treatment on hedonic tendencies (A and B) nor on the number of aggressive acts (C and D, $p > 0.10$ for all groups).

Figure 28. Chronic stress and/or chronic Y5 antagonist treatment produced little change in anxiety behavior. Behavior in the EPM was measured by (A) percent time spent in the open arms, (B) number of open arm entries, and (C) number of times the animal reached the end of the open platform. A two-way ANOVA found a main effect of stress for the number of open arm entries ($p < 0.05$). A two-tailed student's t-test comparing the means between the No Drug groups revealed a small but expected decrease in percent time spent in the open arms ($p < 0.1$), number of open arm entries ($p < 0.05$), and number of times to reach the end of the open arm platform ($p < 0.1$). Although not significant, difference scores (D) obtained from comparing individual pre-experiment and post-experiment anxiety measures showed overall trends in behavior for each group. Significance is illustrated; #, where $p < 0.1$ in a two-tailed student's t-test.

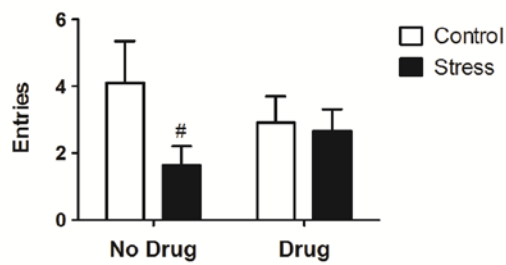
A Percent time in open arms



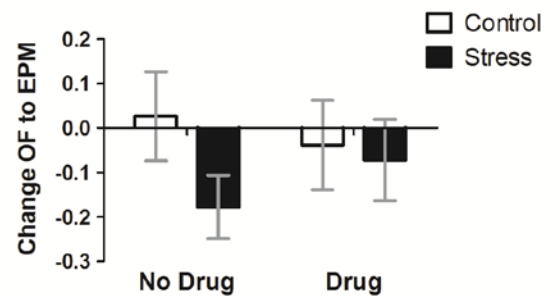
B EPM open arm entries



C EPM entries into end of open arm



D Difference scores



Densitometry was conducted on the autoradiographs generated from the *In situ* hybridization of oligonucleotide probes for NPY and Y5 receptor. Regions of interest were chosen as described in Appendix 1. In the hippocampus, stress induced changes in NPY expression were found through out the *Cornu Ammonis* region. Analysis of NPY mRNA expression in CA1 (Figure 29A) a two-way ANOVA revealed a trend towards main effect of stress, $F(1,27) = 3.08$, $p = 0.0903$) and a stress by drug interaction, $F(1,27) = 4.26$, $p = 0.0489$). Post-hoc comparisons of the No Drug group showed that stress reduced expression ($p < 0.05$, Bonferroni corrected). Similar results were found in region CA2 and CA3a (Figure 29B and C, respectively); there was an significant main effect of stress on NPY mRNA expression, $F(1,27) = 4.55$, $p = 0.0422$ in CA2 and $F(1,28) = 5.92$, $p = 0.0216$ in CA3a, although post hoc analysis found a stress-induced reduction in expression only for the No Drug groups ($p < 0.05$, Bonferroni corrected). In CA3b (Figure 29D), a two-way ANOVA found a trend toward a main effect of stress, $F(1,28) = 3.23$, $p = 0.0832$, and a main effect of stress was found in CA3c (Figure 29E), $F(1,28) = 5.04$, $p = 0.0331$, although post-hoc comparisons were not significantly different for any group ($p > 0.05$ for all groups, Bonferroni corrected). In contrast to the effects found in the CA region, no differences in NPY mRNA expression were found in the upper or lower blade of the dentate gyrus (Figure 29F and G) of these same animals ($p > 0.1$) for all groups. Stress and controls animals in the Drug group did not differ from each other, nor from No Drug controls ($p > 0.05$, Bonferroni corrected, for all analyses).

The mPFC was also examined for NPY mRNA expression. A two-way ANOVA of NPY expression in the prelimbic region (Figure 29H), showed a trend towards a main effect of stress, $F(1,31) = 3.38$, $p = 0.0757$, although post-hoc comparisons were not significantly different ($p > 0.05$ for all groups, Bonferroni corrected). The infralimbic cortex was also examined, however, no differences were found between groups ($p > 0.1$, data not shown).

Analysis of Y5 receptor mRNA expression in the hippocampus found only minor stress and drug related changes. In the upper blade of the dentate gyrus (Figure 30A) a two-way ANOVA found a trend towards a main effect of drug, $F(1,31) = 3.75$, $p = 0.0615$, with no difference between stress and controls in either Drug or No Drug group ($p > 0.05$). In contrast, no effects were found in the lower blade of the dentate gyrus (Figure 30B, $p > 0.01$ for all comparisons). In CA1 (Figure 30C), there was a trend towards a main effect of both stress, $F(1,30) = 2.81$, $p = 0.100$, and drug, $F(1,30) = 2.81$, $p = 0.100$. No differences between stress and controls were found in either the Drug or No Drug groups in the post-hoc comparisons analysis ($p > 0.05$). No alterations in Y5 mRNA expression were found in CA2, CA3a or CA3c (Figure 30D, E and G), $p > 0.10$ for all groups). However, analysis of CA3b (Figure 30F), revealed a significant stress by drug interaction, $F(1,26) = 5.75$, $p = 0.0240$. Post-hoc analysis found a significant stress-related decrease in Y5 expression in the No Drug group ($p > 0.05$, Bonferroni corrected). In the medial prefrontal cortex, there was a trend towards a main effect of stress, $F(1,24) = 3.02$, $p = 0.095$. Post-hoc analysis did not show any differences between stress and controls for either drug group ($p > 0.05$).

Figure 29. Chronic Restraint Stress reduces NPY mRNA expression in the hippocampus, Y5 antagonist treatment appears to partially reverse these effects. NPY mRNA expression was measured in regions of the hippocampus by densitometry. A significant ($p < 0.05$) main effect of stress was found by two-way ANOVA in (B) CA2, (C) CA3a, and (E) CA3c, while a trend ($p < 0.1$) towards a main effect was found in (A) CA1, and (D) CA3b. Post-hoc analyses were conducted between stress and control animals for the No Drug and Drug groups and a significant difference was found where noted. Significance is illustrated; *, where $p < 0.05$. No differences were found between stress and control groups with Drug ($p > 0.05$, Bonferroni corrected). In (H) the Prelimbic region of the mPFC, there was a trend towards a main effect of drug ($p < 0.1$).

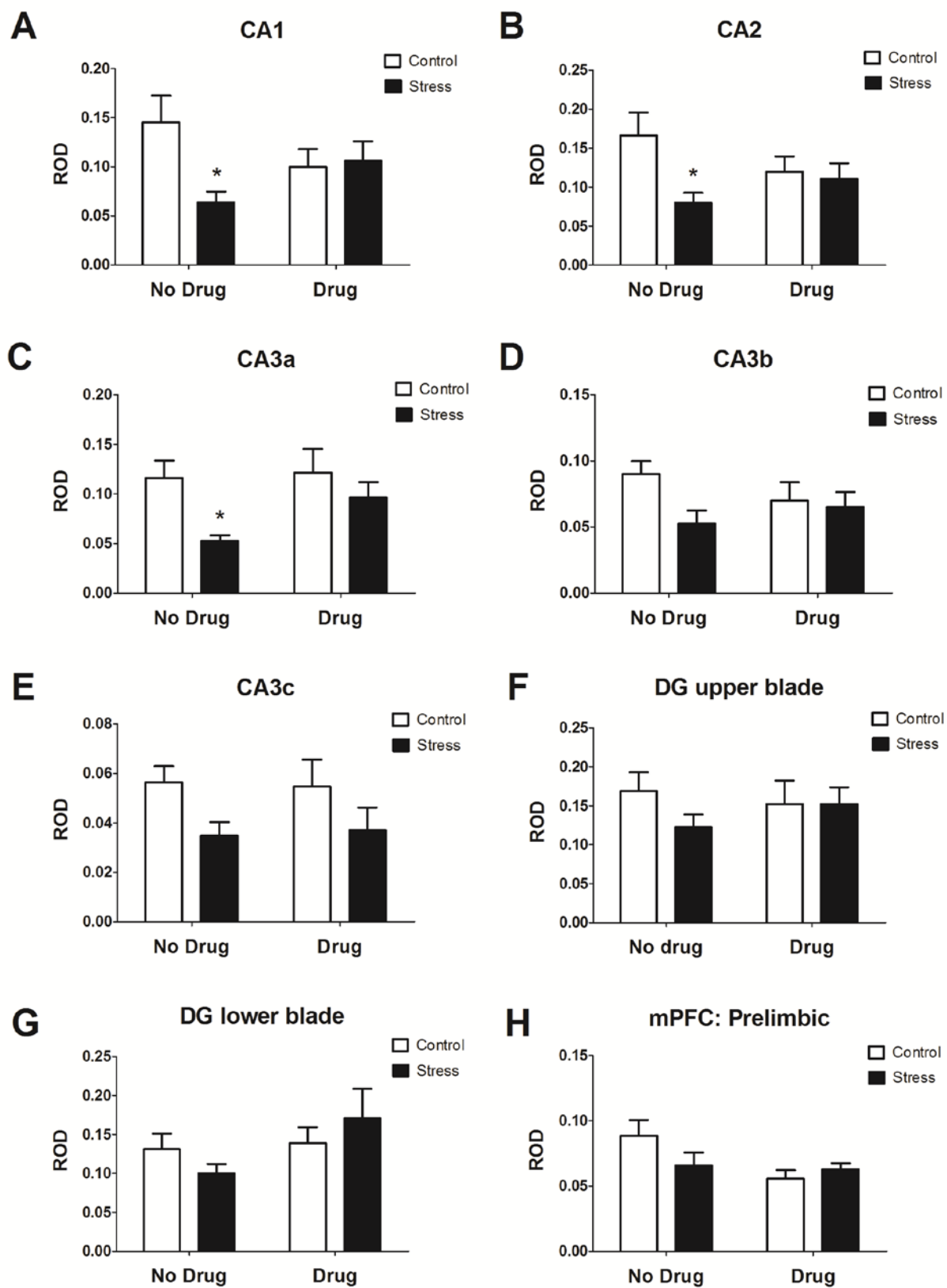
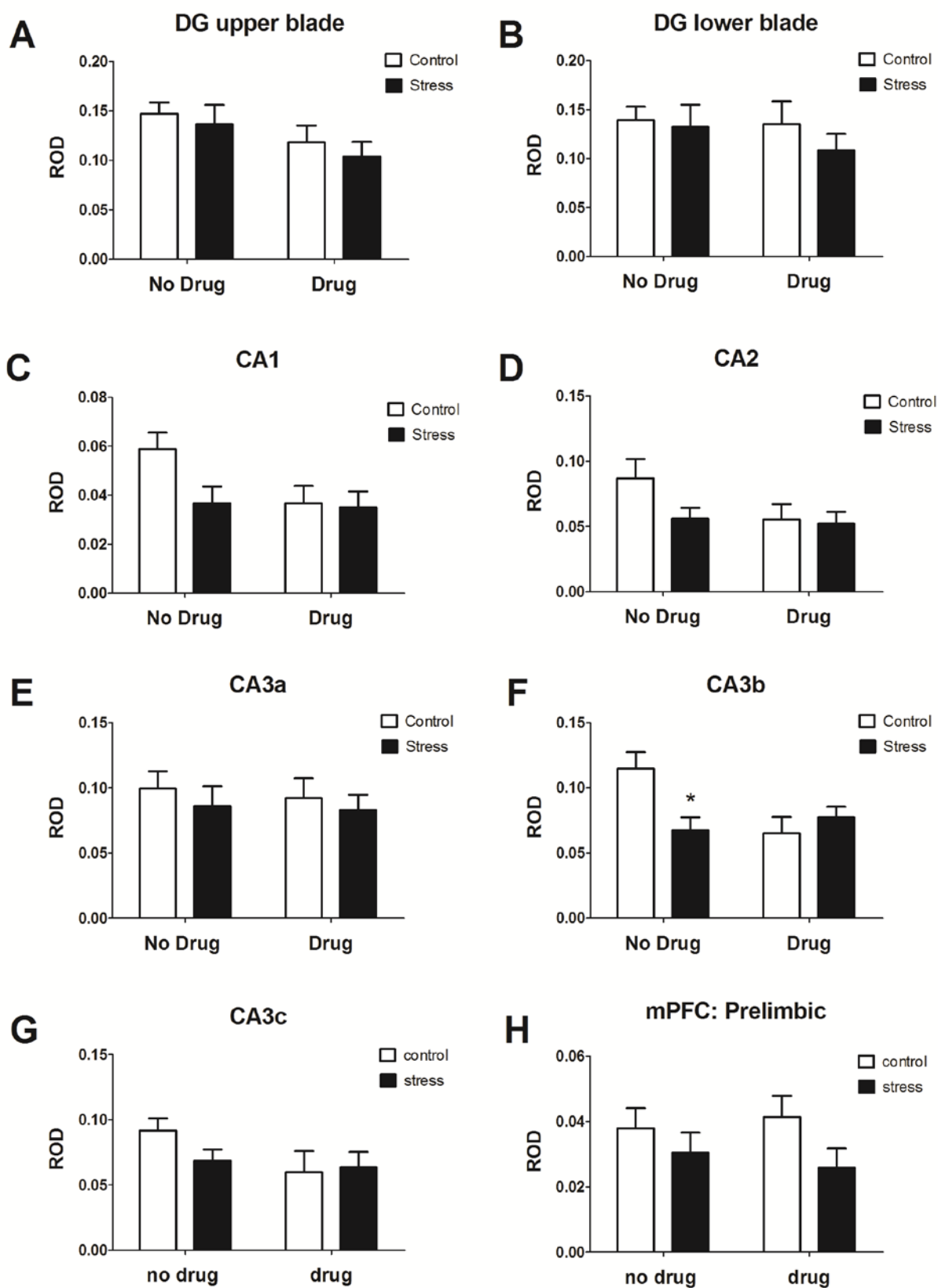


Figure 30. Both chronic restraint stress and Y5 antagonist appear to influence Y5 receptor mRNA expression. Y5 mRNA expression was measured in regions of the hippocampus by densitometry. A trend towards a main effect of stress ($p < 0.1$) was found by two-way ANOVA in (A) the upper blade but not the (B) lower blade of the DG. In the (C) CA1 region, there was a trend towards both a main effect of stress and drug ($p = 0.1$ for both analyses). No effect of stress or drug was found in (D) CA2, (E) CA3a, or (G) CA3c ($p > 0.1$ for all analyses). However, a significant stress by drug interaction was present in (F) CA3b ($p < 0.05$). Post-hoc analysis of CA3b revealed a significant stress-induced decrease in Y5 mRNA expression in the No Drug group. Significance is illustrated; *, where $p < 0.05$. In (H) the Prelimbic region of the mPFC, there was a trend towards a main effect of stress ($p < 0.1$).



Discussion

No differences in anxiety were found after pre-treatment with either CORT or a NMDA receptor antagonist prior to stress. However, no significant differences were found between stress and control groups without drug treatment, so no conclusions can be made about the efficacy of the two treatments. While there was an effect of chronic stress on NPY mRNA expression in the hippocampus, behavioral differences between stress and control groups were not robust enough to achieve significance.

The lack of an effect of both NMDA receptor antagonist and CORT treatment brings up several issues with the design of these experiments. In the first experiment, the necessity for injection of the NMDA receptor antagonist CGP43487 requires that a third, saline injected vehicle group be added to experiment. As the multimodal stressor is not strong enough to induce anxiety symptoms in all individuals, and a 3x2 ANOVA requires more power to achieve statistical significance, we were unable to find behavioral differences even with 18 animals per group. A second issue that may have caused additional variability, was that animals in the treatment groups were given injections. Although injection procedures were practiced without a needle tip in order to habituate the animals to the extra handling, some animals appeared to have more fearful reactions to the injections than others. For this reason, we chose a non-invasive approach to drug administration for the CORT treatment experiment.

There is recent evidence to suggest that even a single CORT injection may be sufficient to cause an increase in anxiety behavior in unstressed animals. Mitra

and Sapolsky (2008) have shown that twelve days after CORT treatment, animals showed decreased exploration into the open arms of the EPM and increased amygdala volume and dendritic hypertrophy in neurons of the basolateral nucleus. Thus, it is possible that the CORT supplementation in the drinking water mimicked some of these stress effects in the control CORT treated animals. Nonetheless, even in non drug-treated treated, non injected animals, with 12-18 animals per group, no behavioral difference was found between stress and control groups for either of these experiments. This was in contrast to previous pilot studies and the results of the conditioned stimulus cued behavioral study (Chapter 5). However, even in this experiment, we were only able to achieve a significant difference between stress and control behavior, when the groups were collapsed across song exposure ($n = 36$ per group).

Since the multimodal stress paradigm produces PTSD-like symptoms only in a fraction of the population of animals subjected to it, this makes it an ideal model for studying stress vulnerability and stress resilience but a poor model for studying the effects of manipulating a candidate neurochemical system using a behavioral endpoint. A model using a more robust stressor, such as CRS has historically produced more consistent behavioral effects (Wood et al. 2008). This paradigm was used in the third experiment, examining the effects of a Y5 receptor antagonist.

Three weeks of CRS significantly reduced weight gain in the stressed groups, regardless of drug treatment. This effect, while consistent with the stress literature (Magarinos et al. 1995; Goldwater et al. 2009), was not necessarily predicted for the drug treatment groups. As the drug was administered orally in the

chow, it was important that animals in the stress and control groups consumed similar amounts of chow in relation to their body weight. As stressed animals continued to gain weight after week 5 of the study, this did not appear to be a factor.

Unfortunately, no in-cage behaviors appeared to be affected by either stress or drug treatment. Anhedonia or decreased sucrose water preference is a common result of chronic stress (Rygula et al. 2005; Kompagne et al. 2008; Tynan et al. 2010). We may not have been able to find this effect for several reasons. First, most experiments have individually housed animals. Therefore, all sucrose consumption can be attributed to a single animal. In this experiment, animals were housed in groups of three and were not separated in order to reduce stressful experiences in the control animals. As all three animals shared a single water bottle, which increased competition, this may have mitigated some of the effect. Second, sucrose preference was tested twice, once on day 2 and again on day 20 of stress. What effect repeated trials may have on sucrose preference is unclear, however, virtually no regular water was consumed when the animals were given the choice of sucrose water on Day 20.

Aggressive acts were also very minimal in the two hours immediately after 6 hours of restraint stress, with matched controls. The protocol used follows the one found in (Wood et al. 2008), which showed significant increases in aggression in the stressed animals after a three- week stress. The only difference was that in this experiment, behavior was recorded using a video camera and no experimenter was present in the room at the time of observation. The majority of animals fell asleep quickly after the start of recording, therefore producing few aggressive acts. It is

possible that having an experimenter in the room was a confounding variable that may have even been a stressful or arousing stimulus that kept the animals awake and induced aggressive behavior. Future studies will examine in cage behavior during the dark cycle when the animals are more likely to be awake.

Finally, although behavior in the EPM differed between stress and control animals in the No Drug group when compared using a unpaired student's t-test, the addition of another variable, and the necessity to compare these groups by a two-way ANOVA diminished this effect. As Sprague-Dawley rats have highly variable pre-stress anxiety behaviors, the combined results of a second behavioral cohort should enhance these results.

Of the stress and drug- induced alterations to the molecular profiles, NPY changed the most significantly. Stress reduced NPY expression in the *Cornu Ammonis* region of the hippocampal formation, which confirms studies of chronic stress in the literature (Sweerts et al. 2001; Sergeev et al. 2005). It appears that Y5 antagonist partially blocks this effect, although there was a trend for the Y5 antagonist to reduce the amount of NPY in the hippocampus of control animals. As many types of NPY receptors can be found in the hippocampus of the rat brain (Parker et al. 1998), it is possible that the balance of these receptors or the amount of NPY protein may be disrupted by the Y5 antagonism.

There appeared to be an interesting stress by drug interaction in the expression of Y5 mRNA. As Lu AA33810 works directly on the Y5 receptors as a competitive antagonist, it was unclear what consequence it would have on Y5 mRNA expression. There was an effect or a trend towards an effect for the drug to

reduce Y5 expression in most sub-regions of the hippocampus, regardless of stress. Although only significant in region CA3b, there was a stress-induced reduction of Y5 mRNA expression. However, if we compared the expression levels of stress and controls in the No Drug group by unpaired two-tailed student's t-tests, there was a significant stress-induced reduction of Y5 expression in all sub-regions of the *Cornu Ammonis*. This would suggest that the drug and stress treatments may have interacted with each other preventing us from seeing a more robust effect. It is of note that Y5 mRNA decreased as a result of both stress and Y5 receptor antagonism, as the aim of this treatment was to reduce the effects of stress on brain and behavior. Although mRNA expression may be reduced with drug treatment, it is unclear how the protein is changing, and how much Y5 receptor is actually available for binding with the ligand.

In light of the above results, and the difficulties in achieving significant differences between non-treatment stress and control groups when multiple comparisons were necessary, this has led us to conclude that the multimodal stress model of PTSD is not effective for use in studies where individual differences are not examined. Individual differences in response to this particular stressor appear to induce variability to the point that we may not be able to see a result of drug treatment unless the drug is 100% effective at preventing anxiety after stress. It is possible, although not practical, that increasing the total number of subjects run in each group may help add power to the analyses. While there was difficulty achieving a significant difference between non-drug groups in the Y5 receptor antagonist study, we believe that a second cohort of animals would be sufficient to

demonstrate stress-induced anxiety in the non-drug groups and show that drug treatment can prevent this effect.

Chapter 7: General discussion and implications for stress research

First and foremost, the results of this research demonstrate that molecular and morphological differences are already apparent in unstressed adult male rats and correlate with basal anxiety levels. In particular, anxious rats have smaller apical dendrites in pyramidal neurons of the mPFC, and anxiety correlates with CART mRNA expression in many regions involved in learning and memory.

Using this information, and, in order to better replicate the parameters of PTSD in humans, where only a percentage of people exposed to a traumatic experience develop PTSD, an animal model of PTSD was developed to produce both stress-vulnerable and stress resilient individuals. When animals were divided into groups based on whether or not there was a stress-induced change in their anxiety levels, differences were found between groups in expression of the neuropeptide CART. Instead of finding molecular changes in the brains of the affected individuals, the PTSD-like group, it was actually the “Resilient” animals that showed differences in CART expression from unstressed controls. These results suggest that stress-resilience is an active process, and that CART may play a role in both neuroprotection and anxiety behavior.

The brain encodes for different aspects of a stressor, including length, timing and modality

Messenger RNA expression of the neuropeptides CART and NPY were differentially affected by both stress timing and modality. Chronic stress had the greatest effect on CART expression, inducing increases in the hippocampus, amygdala and nucleus accumbens and a decrease in the mPFC. This would suggest that a long-term, more severe stressor has larger effects in the brain, which is supported by the chronic stress literature (McEwen 2001; Mitra et al. 2005). In contrast, changes in NPY mRNA expression appeared in the immediate aftermath of an acute stress, increasing in the dentate gyrus and CA3 region of the hippocampus as well as the mPFC. There is evidence that NPY levels in plasma increase over several hours during or after stress (Zukowska-Grojec et al. 1988). As these animals were sacrificed 2 hours after stressor onset, it is possible that NPY has already been upregulated in the brain (Zukowska-Grojec 1995). Additionally, the 21-day chronic restraint stress (see Chapter 6) decreased NPY mRNA levels in the CA1, CA2, and CA3 region of the hippocampus, confirming findings of previously published chronic stress studies (Sergeyev et al. 2005; Zambello et al. 2010). These results suggest a time dependent mechanism for NPY expression. It's possible that the immediate increase in NPY is a homeostatic response to normalize function while the decrease found with chronic stress reflects a maladaptive failure to maintain that compensatory response.

It is important to point out that that many of these changes in mRNA expression are happening 10 days after a single stressful stimulus, a stimulus that

lasts only 10 minutes in the case of COS + 10d. Yet, we are still finding alterations to mRNA expression 10 days later, suggesting that there is a long term change in the protein output for these small neuropeptides even after short and mild stressful experience, and even without a change in behavior. This may be a clue to the phenomenon that people who have experienced prior stress are more prone to develop stress disorders after a subsequent trauma (Sledjeski et al. 2008). These long-term changes in mRNA expression in regions encoding and regulating memories, including fear memories may be involved in priming the brain to respond more quickly or severely to a subsequent stressor.

Individual differences in basal levels of anxiety correlate with morphological differences in rat mPFC.

We have shown that apical dendritic length in pyramidal neurons from layer II/III of the prelimbic (PL) region of the mPFC correlates with anxiety behavior regardless of stress. When unstressed controls were divided into groups that contained individuals from the top and bottom 15% of the anxiety behavior spectrum, those in the least anxious, or Calm group, had significantly longer apical dendrites than the individuals that had the highest anxiety levels. These findings reflect the effects of stress on dendritic morphology in the mPFC. Three weeks of chronic restraint stress causes dendritic retraction and an anxious behavioral profile (Cook et al. 2004; Radley et al. 2004; Brown et al. 2005). A summary of results in the mPFC can be found in Table 1, below.

There were also differences between rat strains. Although all Lewis rats generally spent less time in the center of the open field than Sprague-Dawley (SD)

rats, a sign of anxiety, cells in the PL regions were generally larger. This becomes even more apparent if the lower average body weight of the Lewis strain is taken into account. While we did not account for brain weight, it would be expected to be proportional to body weight in age-matched animals. This phenomenon does not correspond to what we would predict based on strain behavior, and other connections, and even morphology, of the prefrontal cortex needs to be further explored in Lewis rats. Nonetheless, animals from both strains showed differences in mean total apical dendritic length about 15-20% between Calm and Anxious groups.

Additionally, the Sholl analysis revealed a difference in the distribution of dendritic material between the Lewis and SD strains. Calm Sprague-Dawley individuals showed a typical distribution of dendritic material across the dendrite, the largest concentration of branches was located around 90-120 μm from the cell body, which gradually tapered off farther away from the soma. Anxious animals, had similar amounts of dendritic material in the 90-120 μm range, but this tapered off more quickly, resulting a larger reduction of dendritic material in the distal portion of the dendrites as compared to Calm controls. Without the same proportion of inputs from the projections located closest to the pial surface, which tend to be GABAergic horizontal interneurons (Krimer et al. 2001; Cassidy et al. 2010) the most anxious individuals may have a difference in overall connectivity in the mPFC. Lewis rats in both the Calm and Anxious groups had similar Sholl profiles, suggesting that the increase in dendritic length found in the calm animals was spread across the entire dendritic arbor

These results bring up some methodological issues with experiments that measure dendritic length and morphology after experimental treatment. Based on the studies above, there is an approximately 20% difference in mean apical dendritic arbor size between Anxious and Calm controls without any kind of behavioral manipulation. This would suggest that a large part of the variation seen in studies examining dendritic structure of mPFC neurons could be due to this variability if basal anxiety behavior isn't measured and counterbalanced. Many stress studies that have examined mPFC dendritic morphology have assumed behavioral changes without measuring them (Wellman 2001; Radley et al. 2004; Perez-Cruz et al. 2007; Shansky et al. 2009). These results show that individual variability can be a large factor in studies with subject numbers as low as 4-6 per group with the number of neurons traced per animal less than 8, and proposes a strong message against making conclusions about stress-induced dendritic remodeling in mPFC without also measuring behavior in those animals. However, the few studies that have examined post-stress behavior after chronic stress have been able to show enhanced anxiety and/or learning impairment coincide and even correlate with mPFC dendritic morphology (Cerqueira et al. 2005; Liston et al. 2006), suggesting that it is possible to see stress effects on top of the individual differences in these behaviors.

Table 1. A summary of known anxiety and stress-induced changes in mPFC

Stressor	Experiments	Behavior	Morphology	CART	NPY
Basal Anxiety	Current findings	There is a wide range of basal anxiety behavior in the open field and elevated plus maze in SD and Lewis rats	PL (and to a lesser degree IL) apical dendrites are larger in Calm animals Larger apical dendritic length correlates with lower anxiety	Increased expression correlates with higher anxiety	No difference
	Published findings	Wide range of basal anxiety behaviors in SD rats	No published studies	No published studies	Infusion of NPY into PFC produces benzodiazepine like activity (Ehlers, et al. 1997)
Acute Stress including multimodal stress	current findings	No Differences in behavior after AIS + 10d	No changes in dendritic length or spine density after AIS + 10d	AIS +10d - mRNA expression: Trend towards a decrease Multimodal stress - mRNA expression lower in Resilient (PL) But protein expression lowest in PTSD-like (IL)	AIS + 10d and COS +10d- mRNA expression: No differences after a delay COS + 2 hrs – mRNA expression: increased 2hrs after stress No differences between stress resilient and PTSD-like
	published findings	AIS + 10d enhances anxiety behavior (Mitra, et al. 2005)	No published studies	No published studies	No differences immediately or 1 day after restraint stress (Sweerts, et al 2001) Footshock decreases NPY protein expression (Krysiak, et al. 2000) Administration of CRH does not change NPY protein expression
Chronic Stress	Current findings	21d CRS: Some evidence of increased anxiety in elevated plus maze Decreased working memory abilities (Liston, et al. 2006)	PL/IL apical dendrites smaller after 21d CRS, positive correlation with working memory (Liston, et al. 2006)	Significant decrease after 10d CIS	No Change after 10d CIS or 21d CRS
	Published findings	21d CRS increases anxiety behavior 10d CIS increases anxiety behavior (Vyas, et al. 2002)	10 days of mild stress causes dendritic retraction (Brown, et al. 2005) 21 days of CRS causes dendritic retraction (Radley et al. 2004, and Cook et al. 2004) and a reduction in spine density (Radley, et al 2006)	No published studies	No changes in NPY protein levels after 12d restraint (Corder, et al 1992)

What causes individual differences in anxiety?

In examining a group of animals that are the same age, grew up and still live in the same environment, have similar life experiences and a similar genetic profile, particularly in the inbred Lewis rats, it is surprising that we can find such a wide range of anxiety behavior. There are many factors that may influence and enhance anxiety behavior and alter the response to stress. A major factor that we cannot control for is maternal care and behavior during development. The amount of time that mothers lick and groom their pups has been shown to change molecular profiles and connectivity in the amygdala, hippocampus and prefrontal cortex and alter anxiety and stress responses in adult animals (Francis et al. 1999; Meaney et al. 2005; Veenema 2009). For example, adult animals from low-licking mothers show impaired habituation of acoustical startle response and blunted connectivity in the mPFC (Zhang et al. 2005), and shortened dendrites and poorer hippocampal dependent memory (Champagne et al. 2008). Mothers that have been also divided by their anxiety profiles also show differences in the amount of time spent licking and nursing their pups, where those that appeared less anxious actually groomed their pups less (Clinton et al. 2007). This may present both an environmental component, in that a nervous mother may change the anxiety profile of her offspring, and a genetic component, where individual differences in anxiety may be passed on to the offspring. Abusive maternal care has also been modeled by shocking pups in the presence of their mother or during presentation of the mother's scent which suppresses the normal stress hormone response to shock (Moriceau et al. 2006). This type of stressor can cause alterations to HPA axis stress response

in adulthood and maladaptive stress responses (Korosi et al. 2009). However, there is variability in the amount of grooming that each pup receives even from attentive mothers (Felissa van Hasselt, University of Amsterdam, personal communication), as well as differences in consistency of care (Tang et al. 2006), and this may allow for even more individual differences between animals from the same litter or litters with equal amounts of grooming but different schedules.

Stress during development or later on in life can also cause changes to brain and behavior. Maternal separation during early life also has been shown to change the HPA axis responsivity (Anisman et al. 1998; Liu et al. 2000; Aisa et al. 2007) and alter connectivity of brain regions that are involved in stress and reward learning (Gos et al. 2008; Swinny et al. 2009) and enhance depressive-like and anxiety symptoms in adulthood (Huot et al. 2002; Ruedi-Bettschen et al. 2005). However, exposure to novelty during development appears to enhance memory, stress-coping strategies and synaptic plasticity in adulthood (Tang et al. 2003; Akers et al. 2006).

Animal housing and social environment can have profound effects on behavior. Social isolation, a common husbandry practice for male rats has also been shown to have effects on anxiety, reward pathways, cognitive ability, and the response to stress (Ellison et al. 1984; McEwen 2000; Silva-Gomez et al. 2003; Thorsell et al. 2006). However, social defeat, and the hierarchy created within a group of animals that are housed together can also have effects on the brain, anxiety behavior and the levels of circulating stress hormones. Social defeat has shown increased anxiety and a change in sleeping patterns (Kinn et al. 2008), an

reduced activity in the hippocampus and increased depressive-symptoms, which could be reversed by anti-depressants (Rygula et al. 2005; Artola et al. 2006; Miczek et al. 2008). Even subordination within a home cage has effects on basal CORT and testosterone levels and changes in the serotonin system (Blanchard et al. 1993)

Husbandry conditions and experimenter interactions can also have impact on anxiety behavior. Sometimes these issues are identical across all animals in an experiment, but often this isn't the case. We have recently performed an experiment that found that cage changes affected CORT levels and anxiety behavior in the EPM in mice for up to 24 hours after cleaning (Rasmussen, et al. submission in progress). Variability in ambient noise, husbandry staff and housing location can have an effect on the brain. In our facility, we have found differences in the size of prefrontal apical dendrites between two housing locations (Deena Goldwater, Mt. Sinai School of medicine, Supplementary Figure 1). Animals housed in the smaller, quieter Smith Hall had overall larger apical dendrites in mPFC, although a chronic stress effect was found in both facilities. Additionally, there were some behavioral effects from differences in lighting levels based on cage location on a housing cage rack and on the use of sleeping shelters that confirm previously published studies (Balcombe et al. 2004; Van Loo et al. 2004; Izidio et al. 2005). Husbandry practice such as changes in lighting have even been shown to affect nursing profiles of mothers, subsequently enhancing anxiety behavior in adult offspring (Toki et al. 2007); so many of these factors can interact with one another to produce the range of behavioral variability found in laboratory rodents.

It appears that genetics, maternal care, and in-cage and outside prior stressors can all influence the individual differences found in anxiety behavior and the response to stress.

Stress resilience is an active process

Large post-stress differences were found in CART peptide and mRNA expression in the resilient individuals, although not always in the same direction in all brain regions. Differences in the Resilient group were found in areas of the mPFC, amygdala and hippocampus. Each of these regions has been shown to have structural and neurochemical changes as a result of stress (Vyas et al. 2002; Radley et al. 2004), but the above results would suggest that resilience is an active process of preventing these stress-induced effects. In the hippocampus, there is evidence that CART is neuroprotective, and can prevent excitotoxicity and enhance neurogenesis in the hippocampus (Yermolaieva et al. 2001; Wu et al. 2006); this is further discussed below.

Stress resilience appears to be an active process in regards to NPY expression. Higher circulating levels of NPY have been found in humans that have gone through high-stress military survival training (Morgan et al. 2000) and in people recovering from PTSD compared to those with more PTSD symptoms (Yehuda et al. 2006). Administration of NPY into the brain reduces fear potentiated startle and enhances extinction learning (Gutman et al. 2008). These results would suggest that NPY mRNA expression would differ between the Resilient and PTSD-like groups in above experiment. Surprisingly, we found no differences in NPY expression on basal anxiety level or stress using the multimodal model of PTSD.

However, we analyzed mRNA while the published studies examined protein levels. It is possible that the mRNA expression profiles are not predictive of what is happening with the peptide, and future immunohistochemical studies will be performed on the stress resilient and PTSD-like brains.

Evidence that resilience is an active process is also provided by the field of extinction learning. Stress resilience may be thought of as a form of extinction from trauma-induced generalized anxiety, and appears to be a process that happens gradually in a previously stressed animal or person (Yehuda et al. 2006). A high percentage of people have PTSD-like symptoms such as enhanced startle reflex immediately after trauma, but diagnosis of an actual disorder must meet the criteria of being present for more than 3 months after trauma (Yehuda et al. 1998). In PTSD, the gradual extinguishing of fear appears arrested (Milad et al. 2006; Milad et al. 2008). Individual differences have also been found in the ability to extinguish a fear-conditioned response and in the amount of spontaneous recovery seen a population of SD rats (Bush et al. 2007). CART may either play a role or be indicative of other ongoing processes involved in stress resilience through extinction learning pathways.

The benefits and pitfalls of the multimodal stress paradigm

While the multimodal stress paradigm was able to produce significant long-term effects on anxiety behavior with a sufficient number of animals per group (at least 18), a large minority of the stressed individuals (about 20-40%) did not change in their behavior as a result of the experimental manipulations. While this may be a

more applicable model of PTSD in humans, where only a percentage of people exposed to a traumatic experience develop PTSD, most experiments rely on statistical differences between groups to show that an experiment is working. Herein lies the difficulty of using this paradigm in drug treatment studies; Individual differences in response to this particular stressor appear to induce variability to the point that we may not be able to see an effect of drug treatment unless the drug is 100% effective at preventing anxiety after stress.

One method for accounting for this variability is to calculate a change over time from normalized anxiety measures of controls, since even controls have variable anxiety behavior that is predictive of differences in the brain. Another method is to remove some of the variability by excluding individuals who fall on the extreme ends of anxiety behavior, by way of behavioral pre-screening prior to experimental manipulations. However, selecting for “ideal” individuals (those that are not extremely calm or nervous prior to experimental procedures) is a costly and somewhat confounding process.

Yet, choosing to experiment only on “average” individuals could make drug treatment studies irrelevant to a human population. Affected individuals seeking treatment for anxiety and mood disorders are a subset of the population [approximately 31.2% lifetime prevalence for anxiety disorders and 21.4% for mood disorders, as per the National Comorbidity Survey (NCS-R), Kessler et al. 2005]. Comorbidity studies, such as the NCS-R illustrate that there are many factors that increase vulnerability to developing an anxiety disorder, such as childhood experiences (Kessler et al. 1997; Goodwin et al. 2004), previous trauma (Sledjeski

et al. 2008) , and social status (Kessler et al. 2005). It is possible that many of these prior stressors may manifest as subclinical behavioral changes, similar to the range of anxiety behavior seen in laboratory animals. Therefore, it is important to take individual differences into account when using animal models of anxiety and mood disorders, which may necessitate a different way of thinking about and designing experiments.

The role of CART in fear and anxiety behavior

Some of the most interesting findings of this research were that CART neuropeptide mRNA expression correlated with basal levels of anxiety and that CART mRNA and protein levels were altered after stress, but almost entirely in the stress-resilient individuals (See Table 2 for summary). While it is well-established where CART peptide can be found in the brain, little is understood about what CART is doing in the brain or even what CART receptors are and where they are located. This presents a major obstacle when trying to determine the mechanism for the actions of CART pertaining to the production of fear and anxiety behaviors. However, the neuroanatomical circuits and actions of other neurotransmitter systems that lead to elicitation of fear and anxiety behavior are better understood. By combining what little is known about the location and function of CART peptide with what has already been established in these areas, we can infer some of the roles CART could be playing in these regions.

Location and connectivity of CART to other neurotransmitter and hormone systems

In rats, CART peptide is found in amygdala, hippocampus, many regions of the cortex including, the piriform, frontal, prefrontal and orbitofrontal, perirhinal, auditory, and motor cortex, the nucleus accumbens, olfactory processing areas, many hypothalamic nuclei, a few thalamic nuclei, the BNST, periaqueductal gray (PAG), dorsal raphe, locus coeruleus (LC), ventral tegmental area (VTA), medulla, the vagal nerve and spinal cord (Koylu et al. 1998; Murphy et al. 2000). Nearly all of these regions have something in common, they are connected directly or indirectly to amygdala and they have been implicated in the stress response and anxiety behavior (Ottersen 1980, 1981, 1982; Volz et al. 1990; Davis et al. 1993; Petrovich et al. 1996; Van Bockstaele et al. 1999; Adamec et al. 2001; Dong et al. 2001). Although localization of the peptide has not been ascertained in every region, CART has been shown to be a cotransmitter in GABAergic neurons in the nucleus accumbens (Smith et al. 1997) and with epinephrine in the medulla (Wittmann et al. 2004), and many other hormones and peptides in the hypothalamus including corticotrophin releasing hormone (CRH), alpha-melanocyte-stimulating hormone (alpha-MSH), thyrotropin-releasing hormone-(TRH), dynorphin and neurotensin (Elias et al. 2001).

The CART neuropeptide has a direct effect on anxiety behaviors and mood. CART injected into ventricles or CE induces anxiety behavior in the EPM and in a social interaction test (Chaki et al. 2003; Dandekar et al. 2008). Intraventricular CART administration also increased activity in neurons of the LC (Chaki et al.

2003), a region that is both directly and indirectly (through BNST) connected to CE and required for the output of anxiety behavior (Van Bockstaele et al. 1999). Interestingly, injections of CART into the amygdala or lateral ventricles can also decrease depressive-like behavior, such as time immobile during a forced swim test (Dandekar et al. 2009). Projections from CE to the medulla have been shown to control cardiovascular fear responses through the glucocorticoid CRH (Bohus et al. 1996; Salome et al. 2001). Injection of CART into the fourth ventricle induces anhedonia similar to the stress-induced reduction in sucrose consumption (Zheng et al. 2001). The CART positive neurons that are present in the dorsal vagal complex of the medulla have been shown not to be directly involved in food intake (Zheng et al. 2001) but stimulate CRH release (Stanley et al. 2001), which might suggest that they are actually involved in modulating the cardiovascular fear response. Taken together, this evidence points to a role for CART in increasing anxiety and fear behaviors through amygdala signaling. As we found reduced CART protein expression in most regions of the amygdala and BNST in stress-resilient individuals, this would correspond with inhibition of amygdala activity, with CART expression as a direct or indirect result, and an active model of stress resilience.

CART is regulated by glucocorticoids and also is able to modulate stress hormone levels. CART is differentially affected by adrenalectomy in the hippocampus and amygdala. Adrenalectomy reduces CART expression in the dentate gyrus but CE is not affected (Hunter et al. 2007), suggesting different actions of glucocorticoids in these regions. On the other hand, systemic CART administration increases Adrenocorticotrophic hormone (ACTH) and CORT plasma

levels, although intraventricular injection had no effect (Smith et al. 2004). The functions of CART in the brain and the roles it has in modulating stress hormones have been best established in the hypothalamus. CART injection increases CRH and vasopressin in hypothalamus (Smith et al. 2004); and it would not be far leap to suggest that CART could be doing same thing in the amygdala, where both hormones play a role in the stress response. The CE to LC pathway is modulated by CRH, which in turn increases anxiety (Van Bockstaele et al. 1999; Yilmazer-Hanke et al. 2004). Vasopressin has been shown to modulate freezing and cardiovascular stress responses through projections from CE to the LC, PAG and vagal complex of the medulla. While it is not clear whether CART is a cotransmitter with CRH and vasopressin or in other projections that promote the release of these hormones, the above evidence suggests a direct role of CART to increase anxiety behavior through actions on stress hormones. The results from the stress-resilient animals would propose that stress hormones may also be modulating CART expression. It has been shown that those individuals with positive adaptations to stress have a different corticosterone response (Cohen et al. 2006), and this may be influencing the reduction in CART protein found in the stress-resilient amygdala.

Another side of the CART story has been studied in the hippocampus. There is evidence that CART can inhibit voltage-gated calcium channel signaling during cocaine administration in the hippocampus, reducing excitotoxicity (Yermolaieva et al. 2001). More confirmation of the neuroprotective effects of CART can be found in ischemia studies, where blocking CART increased the extent of ischemic damage and CART treatment increased MAPK/ERK signaling (Jia et al. 2008). As we found

higher CART expression in hippocampus of calm and stress-resilient individuals, this would suggest that CART is involved in neuroprotection by reducing stress-induced excitotoxicity. CART has also been shown to increase cell survival in cultured hippocampal neurons through upregulation of BDNF (Wu et al. 2006). In our studies, both calm individuals and stress-resilient animals had higher CART expression in dentate gyrus, which would be consistent with the idea that CART modulates BDNF expression in this region preventing a stress-induced decrease in neurogenesis. This coupled with the above evidence suggests a neurotrophic or neuroprotective role for CART in the hippocampus. These relationships can form the basis of future studies of individual differences in neurogenesis and stress effects on structural plasticity in hippocampus.

Table 2. A summary of CART mRNA and protein expression findings in the brain.

Summary of CART Expression Findings

Behavior	Expression	Hippocampal Formation	Central Nucleus Amygdala	Medial Nucleus Amygdala	Other Amygdala	Medial Prefrontal Cortex	Orbitofrontal Cortex
Basal Anxiety	mRNA	Increased in Calm Individuals	No difference	Increased in Calm Individuals	Not known	No difference	No difference
	mRNA correlations	Increased expression correlates with lower anxiety	Increased expression correlates with higher anxiety	No correlation	Not known	Increased expression correlates with higher anxiety	Trend that increased expression correlates with lower anxiety
Stress Response	mRNA	Increased in Resilient Individuals	Increased in Resilient Individuals	No difference	Not known	Decreased in Resilient Individuals (PL)	No difference
	Protein	Increased in Resilient Individuals	No difference	Decreased in Resilient individuals	Decreased in Resilient Individuals (LA, BLA, BNST)	Trend towards decrease in resilient individuals. Significant decrease in PTSD-like	No difference

CART has multiple functions in the brain

The above evidence indicates that CART neuropeptide has at least two roles in brain, which are summarized in Table 3. In regions of adult neurogenesis like the hippocampus, it appears that CART is neuroprotective, inhibits excitotoxicity, and that it also may modulate important trophic factors for neurogenesis. This would support our findings of higher CART expression in both calm unstressed and stress-resilient animals. Stress has been shown to reduce hippocampal neurogenesis, and treatments that increase neurogenesis prevent stress-induced changes in anxiety behavior and memory (Pham et al. 2003; McEwen et al. 2004; Kasper et al. 2008).

In the amygdala, CART appears to have a direct effect on behavior. It is anxiogenic, which also is supported by the lower protein expression found throughout amygdala and BNST in the stress-resilient animals. There is one major caveat to this result, variable effects on CART expression were found in the CE (and to a lesser degree ME) with anxiety behavior of unstressed controls and in stress-resilient individuals. In unstressed controls, higher CART mRNA expression correlated with increased anxiety in CE. This result appears to correspond with the general anxiogenic effects of CART expression. However, after stress, animals that were resilient had higher CART mRNA expression in CE but no differences were found in protein expression. Like the rest of the amygdala nuclei, there was lower protein expression in ME in the resilient animals, however, calm individuals also had higher CART mRNA expression in ME. There is some evidence that these amygdala nuclei are responsible for encoding different aspects of stress. ME is involved in integrating odor information and may be necessary to produce

appropriate social and sexual responses (Newman 1999), and CART has been found in ME neurons that also respond to odors from conspecifics (Donato et al. 2010). In fact, ME may be more responsible for predator odor conditioning and defensive behaviors than BLA or CE (Blanchard et al. 2005; Takahashi et al. 2005). In addition, chronic immobilization stress induces dendritic hypertrophy in BLA and BNST, without affecting neurons in CE (Vyas et al. 2003); this is similar to where we find changes in CART expression. Further description of the multiple pathways through CE can be found below.

The medial prefrontal cortex also shows differential effects on CART expression between basal anxiety levels and stress-induced anxiety. In unstressed animals, higher CART expression also correlated with increased anxiety. This corresponded with the decrease in PL CART mRNA expression found in the stress-resilient group, however, a decrease in CART protein expression was found in the IL region in all stressed animals, although this was only significant in the PTSD-like group. CART mRNA expression in the mPFC was generally very low. In the protein analysis, although there were many CART immunolabeled fiber tracts, few immunolabeled cell bodies were found. This would suggest that the population of cells analyzed by mRNA expression is different than the population of CART-positive fibers analyzed for the protein expression. Although it is not clear whether CART activity in this region is excitatory or inhibitory, these results appear to go in the same direction; CART mRNA is higher with increased anxiety, and lower in stress-resilient animals. CART protein is reduced in the mPFC of PTSD-like

individuals, but these are projections that are coming from somewhere else, perhaps the amygdala.

The central amygdala and the mPFC are key regions responsible for the production of fear conditioning and fear extinction behaviors (Nader et al. 2001; Quirk et al. 2003), and may have two systems modulating them; one basal system that mediates general anxiety levels under normal circumstances, and a secondary system that is only responsive during stressful situations, or during the recall of these situations. Evidence for this can be found in the central amygdala, where there are connections from CE to PAG that are involved in fear conditioned freezing and fear-potentiated startle (LeDoux et al. 1988) and two pathways have identified between CE and the LC, a region necessary for elicitation of fear-conditioned freezing and general anxiety (Van Bockstaele et al. 1999). The direct connection from CE to LC is regulated by the glucocorticoid CRH and well as dynorphin and norepinephrine. This pathway is responsible for fear-conditioned freezing. In the second pathway, neurons from CE project first to BNST, which then projects to LC. This pathway has been shown to be more responsible for anxiety behavior, such as the amount of time spent in the open arms of the EPM. Since this was the measure that was used to differentiate PTSD-like and resilient individuals, we would expect that this pathway would be the one affected, and that seems to be the case; CART expression is reduced in BNST. However, we do not know whether direct fear behavior has been altered in our animals. In CE, CART mRNA expression was higher in the Resilient group, but protein levels were unchanged. It is possible that

the expression patterns we found are not related to the anxiety pathway and that the ability to respond to an unconditioned stimulus is similar in all animals.

In the mPFC, there is evidence that PL and IL may have different functions and connections. The IL mainly connects to CE while PL connects to several other amygdalar regions including LA and CE (McDonald et al. 1996). This may be indicative of a different role for each of these regions in the inhibition of the amygdala. A summary of the regions with conflicting CART evidence can be found in Table 4.

Table 3. The functional implications of CART peptide expression in regions with consistent expression profiles

The functional implications of CART peptide expression

Region	Finding	Findings in the literature	Implications
Hippocampus	<p>CART mRNA higher in calm unstressed animals</p> <p>CART mRNA and protein higher in stress resilient animals compared to PTSD-like</p>	<p>CART shown to prevent hippocampal excitotoxicity after ischemia</p> <p>CART necessary for cell maturation of newly born neurons in hippocampal cultures</p>	<p>CART militates against the deleterious effects of stress in the hippocampus - possibly neuroprotective</p>
Amygdala	<p>Higher CART mRNA correlates with anxiety in unstressed animals</p> <p>CART protein lower in resilient animals in ME, BLA, LA and BNST</p>	<p>CART is anxiogenic when injected into the amygdala</p> <p>CART is anxiogenic when injected into the locus coeruleus, which receives direct and indirect input from amygdala</p>	<p>CART has anxiogenic properties in some nuclei of the amygdala, and must be actively reduced to prevent anxiety after stress</p>

Table 4. Possible implications of CART expression in regions with conflicting expression.

Regions with conflicting evidence

Region	Finding	Findings in the literature	Implications
Amygdala Central Nucleus	<p>Higher CART mRNA correlates with anxiety in unstressed animals</p> <p>CART mRNA higher in Resilient animals</p> <p>No difference in protein in stressed animals</p>	<p>CART is anxiogenic when injected into CE</p> <p>CART is anxiogenic when injected into the locus coeruleus, which receives direct and indirect input from CE</p> <p>CE may not be as responsive to predator odor stress</p>	<p>Basal: CE (and CART activity in CE) increase anxiety</p> <p>Stress: CE activity maybe resistant to change in CE even in resilient group or predator stress may not involve CE</p>
Amygdala Medial Nucleus	<p>CART mRNA higher in Calm animals</p> <p>No difference in CART mRNA in stressed animals</p> <p>CART protein lower in Resilient animals</p>	<p>ME responsive to predator odor stress and mating odor cues</p> <p>CART found in ME neurons that also respond to conspecific odors and may be involved in sexual behaviors</p>	<p>Basal: CART may have roles other than anxiety in this region</p> <p>Stress: ME may be very responsive to predators stress</p>
Medial Prefrontal Cortex	<p>Higher CART mRNA correlates with anxiety in unstressed animals</p> <p>CART mRNA lower in Resilient animals</p> <p>CART protein is decreased in all stress groups but only significant in the PTSD-like group</p>	<p>CART is most commonly co-localized in GABA positive neurons</p> <p>Mostly CART positive fiber tracts in mPFC</p> <p>mPFC exerts inhibitory control on amygdala during fear extinction</p>	<p>Basal: CART activity in mPFC may increase anxiety</p> <p>Stress: Decreases in CART protein may be from projections from other regions of the brain</p>

Future directions and conclusions

This research with the neuropeptide CART presents a novel mechanism involved in both PTSD and anxiety disorders. There may be immediate translational relevance, in that plasma CART screening in humans may be telling of resilience after trauma or indicative of anxiety problems. The fact that different brain regions may have different roles for CART makes this peptide a difficult target for drug studies, but extremely compelling. It appears that CART is beneficial for the hippocampus, so any systemic drug that reduces CART in the amygdala may have unwanted side effects that are related to learning and memory or even depression.

It is still unknown whether CART is directly affecting behavior or if it is consequently responding to another signal that causes both behavioral change and a change in CART expression. Evidence from the literature and the ability of CART injections to alter anxiety would point to CART having a direct effect on behavior. Future studies will manipulate CART levels, either by increasing them with injection of peptide or using siRNA to block CART peptide translation. This will have to be done in a region specific manner; in the hippocampus and mPFC, and later in CE to see first what effects it has on basal anxiety, and then what effect it has on stress. An interesting study would be to compare CART effects in BNST and CE, to better understand what role CART is playing in each region. This may help to pull apart the basal anxiety effects from the stress-induced changes.

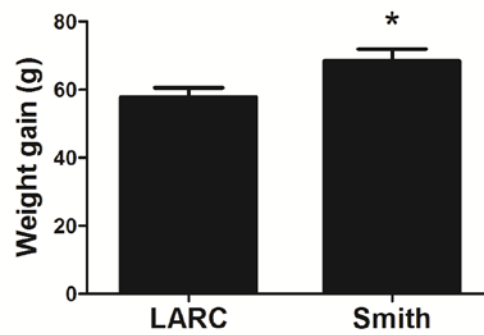
The most important message of these studies is that individual differences need to be taken into account when doing any behavioral experiment, but particularly in stress research. The apparent structural and molecular differences between the top

and bottom 20% of control individuals are very similar to what results are found in a stress study. The mPFC, in particular, is very labile and quick to respond to environment influences. Using small groups of animals in morphological experiments could unintentionally produce incorrect or biased results based on individual differences and not on the actual experimental manipulations.

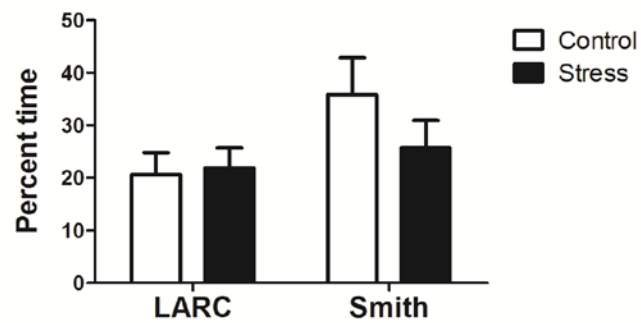
Another theme of this research has been that a better understanding of stress resilience may be the key to understanding PTSD and how to treat it. Animal models of PTSD that cause behavioral changes or anxiety in the vast majority of the experimental group may be missing the chance to look at the changes that are occurring in the brain in a well-adapted animal. It appears that for anxiety behavior to remain unchanged in the stress-challenged individual, many active processes must occur. These findings provide support for the direction of medicine towards individualized treatments based on genetic profile and current brain chemistry for PTSD and all types of anxiety and mood disorders.

Supplementary Figure 1. Effects of husbandry on weight gain, anxiety and dendritic morphology in the mPFC. Identical chronic restraint stress experiments were performed in two facilities on campus. (A) Control animals showed a larger weight gain in the smaller, quieter Smith Hall facility compared to the larger LARC facility. (B) A two-way ANOVA showed a trend towards a main effect of facility on anxiety behavior in the EPM. (C) A Sholl analysis of total apical dendritic material of pyramidal neurons in the mPFC revealed large dendrites in the controls from Smith Hall compared to control animals from the LARC facility.

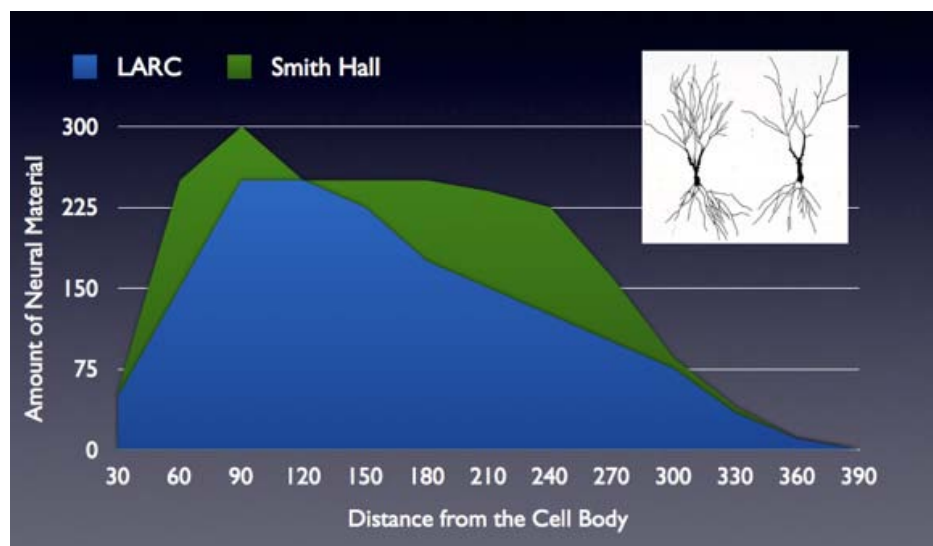
A 10 day Weight gain LARC v Smith Controls



B CIS: percent time in open arms
LARC vs. Smith



C



Appendix 1: Regions of interest used for densitometry

When analyzing autoradiographs of the dentate gyrus of the hippocampus (Supplementary Figure 2A), only the granule cell layer was clearly defined.

Densitometry measurements for this region were examined at Bregma -2.8mm and Bregma -3.3mm. When no difference between these areas was found, a mean was calculated for further analysis. In slides processed for NiDAB immunohistochemistry, the layers of the hippocampal formation were able to be defined with more detail (See Figure 19 for all NiDAB immunohistochemical regions). The dentate gyrus of NiDAB stained sections was further divided into the molecular layer, granule cell layer, granule cell/hilar border, and hilus. Each region was analyzed separately.

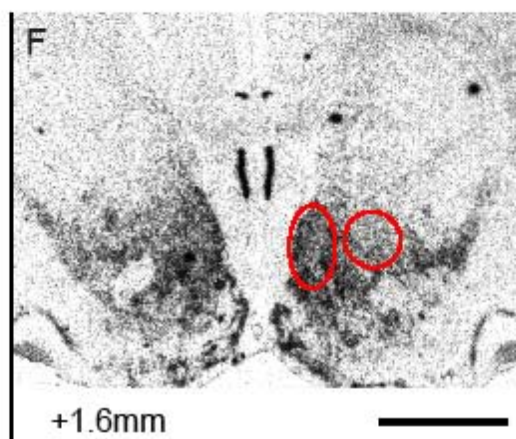
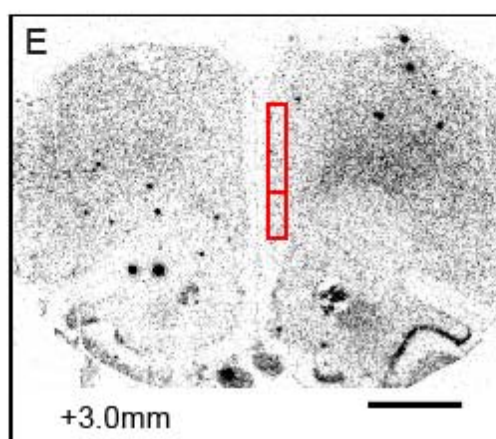
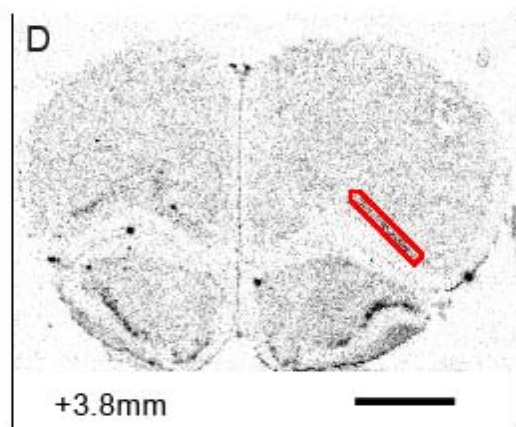
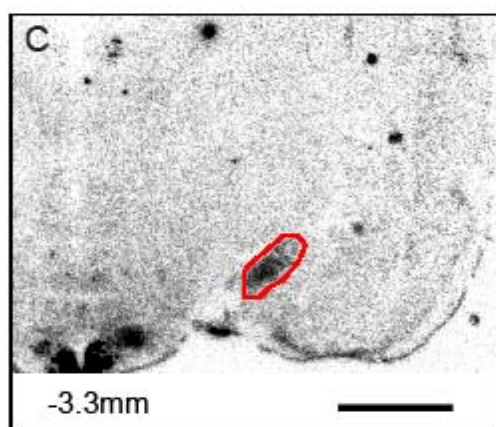
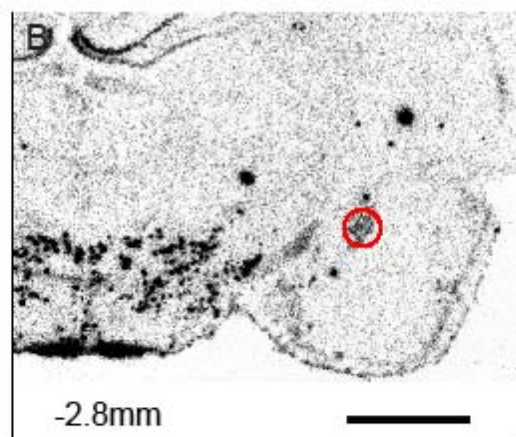
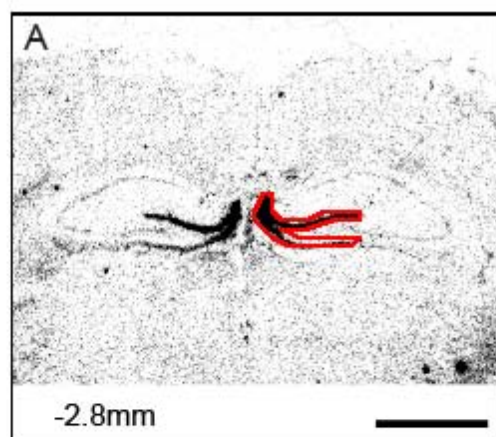
The *Cornu Ammonis* region of the hippocampus was divided into five subregions based on atlas landmarks (Paxinos et al. 1998); CA1, CA2, and CA3, which was further divided into 3 parts (see Figure 19A). CA3a comprised the lateral curved edge of the CA region, as seen in coronal sections. This area was further divided into the pyramidal layer and the stratum lucidum, a region known to have a high concentration of mossy fiber terminals. CA3b comprised the region of CA3 between the curved lateral edge and the lateral edge of the dentate gyrus. CA3c contained the pyramidal cell layer of CA that is found in between the top and bottom blades of the dentate gyrus, and is adjacent to the hilus. In autoradiographs, only the pyramidal layer of CA is visible. In the NiDAB immunolabeled sections, both the pyramidal layer and the stratum lucidum could be analyzed.

The amygdala was divided into several nuclei based on atlas land marks. For both autoradiographs and NiDAB immunolabeled sections, the central nucleus of the amygdala (Supplementary Figure 2B) was analyzed at both an anterior (Bregma -2.6mm) and posterior (Bregma -3.1mm) level. Where there was no difference between anterior and posterior, the mean of these areas was calculated and used for further analysis. The medial nucleus of the amygdala (Supplementary Figure 2C) was divided into three regions based on shape and location; anterior (Bregma -2.6mm), middle (Bregma -3.1mm) and posterior (Bregma -3.6mm). As an anterior to posterior expression gradient was found for CART peptide and mRNA expression, these regions were both analyzed separately and averaged together. At the same level as measured in the medial nucleus, densitometry measurements for the basolateral and lateral nucleus of the amygdala were also collected.

In the prefrontal cortex, layer II/III of the orbitofrontal cortex was analyzed at Bregma +3.8mm (Supplementary Figure 2D), as the highest expression levels of CART appeared around this region. In the medial region of the prefrontal cortex, layer II/III was further divided into prelimbic and infralimbic regions based on landmarks (Supplementary Figure 2E). When available, three regions for each brain were sampled throughout the mPFC, an anterior (Bregma = +3.3mm), middle (Bregma = +3.0mm) and posterior (Bregma = 2.7mm). As there were generally no differences in densitometry measurements for these regions, they were averaged together, except as noted.

The nucleus accumbens was sampled at Bregma = +1.6mm based on previous research (Hunter et al. 2005), and divided into a shell and core region based on atlas landmarks (Supplementary Figure 2F).

Supplementary Figure 2. Autoradiograph examples of regions sampled by densitometry for mRNA expression. Regions were sampled as defined by the red outlines for (A) upper and lower blades of the dentate gyrus of the hippocampal formation; (B) central nucleus of the amygdala; (C) medial nucleus of the amygdala; (D) orbitofrontal cortex; (E) Prelimbic and Infralimbic regions of the medial prefrontal cortex and (F) the shell and core of the nucleus accumbens.



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