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POPPIES AND PTSD: OPIOID INFLUENCE ON A PRECLINICAL MODEL OF POSTTRAUMATIC STRESS DISORDER.

Sarah Vunck
Virginia Commonwealth University

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POPPIES AND PTSD:
OPIOID INFLUENCE ON A PRECLINICAL MODEL OF
POSTTRAUMATIC STRESS DISORDER.

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University

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Virginia Commonwealth University, 2009

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# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
<td>ii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>vii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>viii</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>x</td>
</tr>
<tr>
<td>Abstract</td>
<td>xiii</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Posttraumatic stress disorder, its neurobiology and its current treatment</td>
<td>1</td>
</tr>
<tr>
<td>Posttraumatic Stress Disorder Criteria</td>
<td>2</td>
</tr>
<tr>
<td>Neurobiology of PTSD</td>
<td>5</td>
</tr>
<tr>
<td>The Hypothalamic-Pituitary-Adrenal axis</td>
<td>5</td>
</tr>
<tr>
<td>Hypothalamic-Pituitary-Thyroid Axis</td>
<td>8</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>9</td>
</tr>
<tr>
<td>Amygdala</td>
<td>10</td>
</tr>
<tr>
<td>Prefrontal Cortex</td>
<td>11</td>
</tr>
<tr>
<td>Catecholamines</td>
<td>12</td>
</tr>
<tr>
<td>Serotonin</td>
<td>14</td>
</tr>
<tr>
<td>GABA/Benzodiazepine Receptor System</td>
<td>16</td>
</tr>
<tr>
<td>Glutamate/NMDA Receptor System</td>
<td>16</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>17</td>
</tr>
<tr>
<td>Opioids</td>
<td>18</td>
</tr>
<tr>
<td>Psychological Approaches to the Treatment of PTSD</td>
<td>21</td>
</tr>
<tr>
<td>Prolonged exposure</td>
<td>21</td>
</tr>
<tr>
<td>Cognitive processing therapy</td>
<td>22</td>
</tr>
<tr>
<td>Eye movement desensitization and reprocessing</td>
<td>22</td>
</tr>
<tr>
<td>Stress inoculation training</td>
<td>23</td>
</tr>
<tr>
<td>Exposure therapy using virtual reality</td>
<td>23</td>
</tr>
<tr>
<td>Relaxation training</td>
<td>23</td>
</tr>
</tbody>
</table>
Cognitive behavioral group therapy. ................................................................. 24
Preclinical Models of PTSD .............................................................................. 24
  Neurobiological systems models of PTSD. ..................................................... 25
  Genetic model approaches to PTSD. ............................................................ 25
Trauma/Stress initiated models of PTSD. ......................................................... 25
  Mechanism based models of PTSD. ............................................................. 26
Rationale .......................................................................................................... 28
Methods Experiment I ..................................................................................... 30
  Subjects ........................................................................................................ 30
  Drugs .......................................................................................................... 30
    Choice of drugs and doses tested .............................................................. 31
  Apparatus ..................................................................................................... 32
  Procedure ..................................................................................................... 33
  Data Analysis ............................................................................................... 36
Results Experiment I ....................................................................................... 37
  Preliminary studies for environment one ..................................................... 37
    Pilot Group. .............................................................................................. 37
    JDtic and Control Groups. ........................................................................ 38
    NorBNI and Control Groups. .................................................................. 40
    Noise disturbances and stress assays ...................................................... 42
  Control studies from environment two ....................................................... 43
  Control data for environment two ............................................................... 45
    Fear control group. .................................................................................. 45
    Kappa Agonist Groups (acute). ................................................................. 46
    Kappa Opioid Receptor Antagonist (acute). ............................................. 49
    Benzodiazepine/GABAA agonist ............................................................... 51
    Kappa Opioid Agonist Group (7 days after administration). .................... 52
Discussion Experiment I ................................................................................. 54
  Pilot and control data from environment one ............................................. 54
  Acute kappa modulation of fear behavior ................................................. 55
  Acute benzodiazepine modulation of fear behavior .................................... 56
List of References ....................................................................................................................... 105
Vita.............................................................................................................................................. 129
List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1. Fear conditioning literature survey of methods</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Table 2. Fear conditioning summary of results</td>
<td>87</td>
<td></td>
</tr>
</tbody>
</table>
**List of Figures**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.</td>
<td>Fear Conditioning Pilot group.</td>
<td>38</td>
</tr>
<tr>
<td>Figure 2.</td>
<td>Contextual Fear Conditioning JDtic and Control groups.</td>
<td>39</td>
</tr>
<tr>
<td>Figure 3.</td>
<td>Cue Fear Conditioning JDtic and Control groups.</td>
<td>40</td>
</tr>
<tr>
<td>Figure 4.</td>
<td>Context Fear Conditioning NorBNI and Control groups.</td>
<td>41</td>
</tr>
<tr>
<td>Figure 5.</td>
<td>Cue Fear Conditioning NorBNI and Control groups.</td>
<td>42</td>
</tr>
<tr>
<td>Figure 6.</td>
<td>Fear Conditioning Control groups.</td>
<td>45</td>
</tr>
<tr>
<td>Figure 7.</td>
<td>Fear Conditioning Enadoline groups.</td>
<td>46</td>
</tr>
<tr>
<td>Figure 8.</td>
<td>Enadoline Open Field.</td>
<td>47</td>
</tr>
<tr>
<td>Figure 9.</td>
<td>Fear Conditioning U50,488 groups.</td>
<td>48</td>
</tr>
<tr>
<td>Figure 10.</td>
<td>U50,488 Open Field.</td>
<td>49</td>
</tr>
<tr>
<td>Figure 11.</td>
<td>Fear Conditioning NorBNI groups.</td>
<td>50</td>
</tr>
<tr>
<td>Figure 12.</td>
<td>NorBNI Open Field.</td>
<td>50</td>
</tr>
<tr>
<td>Figure 13.</td>
<td>Fear Conditioning Diazepam Groups.</td>
<td>51</td>
</tr>
<tr>
<td>Figure 14.</td>
<td>Diazepam Open Field.</td>
<td>52</td>
</tr>
<tr>
<td>Figure 15.</td>
<td>Enadoline Open Field.</td>
<td>53</td>
</tr>
<tr>
<td>Figure 16.</td>
<td>U50,488 Open Field.</td>
<td>54</td>
</tr>
<tr>
<td>Figure 17.</td>
<td>Enadoline Contextual Fear.</td>
<td>68</td>
</tr>
<tr>
<td>Figure 18.</td>
<td>Enadoline Activity Data.</td>
<td>69</td>
</tr>
<tr>
<td>Figure 19.</td>
<td>U50,488 Contextual Fear.</td>
<td>70</td>
</tr>
<tr>
<td>Figure 20.</td>
<td>U50,488 Activity Data.</td>
<td>71</td>
</tr>
</tbody>
</table>
Figure 21. NorBNI Contextual Fear ................................................................. 72
Figure 22. NorBNI Activity Data ................................................................. 73
Figure 23. Enadoline Extinction Time Course ............................................. 74
Figure 24. NorBNI Extinction Time Course ................................................. 75
Figure 25. NorBNI+ Enadoline Contextual Fear ........................................ 76
Figure 26. NorBNI+ Enadoline Contextual Fear ......................................... 77
Figure 27. NorBNI + Enadoline Activity Data ............................................ 78
Figure 28. Fentanyl contextual fear ............................................................... 79
Figure 29. Fentanyl Activity Data ................................................................. 79
Figure 30. Morphine Contextual Fear .......................................................... 80
Figure 31. Morphine Activity Data ............................................................... 81
Figure 32. Naloxone Contextual Fear ........................................................... 82
Figure 33. Naloxone Activity Data ............................................................... 82
Figure 34. Naloxone + Morphine Acute Contextual Fear ......................... 83
Figure 35. Morphine+ Naloxone Extinction of Contextual Fear ..................... 84
Figure 36. Morphine + Naloxone Activity Data .......................................... 85
Figure 37. Buprenorphine Contextual Fear .................................................. 86
Figure 38. Buprenorphine Activity Data ...................................................... 86
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>serotonin or 5-hydroxytryptamine</td>
</tr>
<tr>
<td>ACC</td>
<td>anterior cingulate cortex</td>
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<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
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<td>APA</td>
<td>American Psychological Association</td>
</tr>
<tr>
<td>BZ</td>
<td>benzodiazepine</td>
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<tr>
<td>cm</td>
<td>centimeter</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CRF</td>
<td>corticotropin-releasing factor</td>
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<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
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<tr>
<td>DA</td>
<td>dopamine</td>
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<tr>
<td>dBA</td>
<td>decibels</td>
</tr>
<tr>
<td>DOR</td>
<td>delta opioid receptor</td>
</tr>
<tr>
<td>DSM</td>
<td>Diagnostic and Statistical Manual of Mental Disorders</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
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<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HPA axis</td>
<td>Hypothalamic-Pituitary-Adrenal axis</td>
</tr>
<tr>
<td>HPT axis</td>
<td>Hypothalamic-Pituitary-Thyroid Axis</td>
</tr>
<tr>
<td>ITI</td>
<td>intertrial intervals</td>
</tr>
<tr>
<td>JDtic</td>
<td>(3R)-7-hydroxy-N-((1S)-1-[[3R,4R]-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl]-2-methylpropyl)-1,2,3,4-tetrahydro-3-isoquinolinecarboxamide</td>
</tr>
</tbody>
</table>
KOR  kappa opioid receptor
LC  locus ceruleus
LTP  long term potentiation
m  minute
MAPK  mitogen-activated protein kinase
MOR  mu opioid receptor
mPFC  medial prefrontal cortex
MRS  magnetic resonance spectroscopy
NAA  N-acetyl aspartate
NAc  nucleus accumbens
NE  norepinephrine
NIR  near infrared light
NMDA  N-methyl-D-aspartate
norBNI  norbinaltorphimine
NPY  neuropeptide Y
PFC  prefrontal cortex
PFC  pavlovian fear conditioning
PNS  peripheral nervous system
POMC  pro-opiomelanocortin
PTSD  posttraumatic stress disorder
s  second
SPECT  single photon emission computerized tomography
SSRI  selective serotonin re-uptake inhibitor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3</td>
<td>tri-iodothyronine</td>
</tr>
<tr>
<td>T4</td>
<td>thyroxine</td>
</tr>
<tr>
<td>TRH</td>
<td>thyrotropin-releasing hormone</td>
</tr>
<tr>
<td>TSH</td>
<td>thyrotropin stimulating hormone</td>
</tr>
<tr>
<td>VA</td>
<td>Veterans Administration</td>
</tr>
</tbody>
</table>
Abstract

POPPIES AND PTSD: OPIOID INFLUENCE ON A PRECLINICAL MODEL OF POSTTRUMATIC STRESS DISORDER.

By Sarah Anne Vunck, M.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2012.

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Posttraumatic Stress Disorder (PTSD) is an anxiety disorder that affects over 7.7 million adults and carries an estimated societal cost of $3.1 billion every year. People develop PTSD after exposure to a traumatic event. Alone or combined, approved pharmacotherapies or psychotherapy are somewhat effective, but symptoms for many remain refractory. Emerging evidence suggests that opiate systems may modulate the development and expression of PTSD, and their role can be investigated preclinically. Pavlovian fear conditioning is a preclinical model which elicits behaviors mirroring those that occur in humans during and after exposure to trauma. This presents an experimental tool that can help elucidate the opiate mechanisms involved in traumatic memory as well as the resulting fear behavior.
Mu opioid receptor (MOR) analgesics, such as morphine, are often given as a response to trauma, and there is emerging evidence that they are, at least partially, protective against PTSD. The kappa opioid receptor (KOR) system has also been implicated in stress-related processes, with KOR agonists reported to enhance stress in both laboratory animals and in humans, and KOR antagonists reported to attenuate stress-like behaviors preclinically. This project attempted to clarify part of the role of the mu and kappa opiate receptor systems in mediating effects of Pavlovian fear conditioning in mice as a predictor of their involvement in some of the signs and symptoms of PTSD.

Kappa agonists increased acute fear responses but surprisingly also facilitated fear extinction learning. This would suggest that the use of kappa agonists might increase the efficiency and effectiveness of this therapy and could improve existing PTSD patient outcomes. MOR agonists, as well as KOR antagonists reduced acute and long-term fear behavior. These results support that the use KOR analgesics like morphine and fentanyl in the treatment of trauma could have an added benefit of reducing the emergence and persistence of PTSD. Self-medication may help explain the comorbidity of opioid abuse in PTSD patient populations. Understanding the relative effects of these opiate ligands could lead to more informed usage of MOR analgesics which vary in mu and kappa receptor activity under battlefield and other traumatic conditions.
Poppies and PTSD: Opioid influence on a preclinical model of posttraumatic stress disorder.


Posttraumatic Stress Disorder (PTSD) currently affects over 7.7 million adults in the U.S. and prevalence is increasing (Kessler et al., 2005; Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995). PTSD treatment can involve psychotherapy and pharmacotherapy. The most effective PTSD psychotherapy is exposure based behavioral therapy (Hetrick, Purcell, Garner, & Parslow, 2010). This method involves the exposure of the patient to aversive stimuli (real or simulated) under controlled conditions. The two pharmacotherapies approved by the FDA to treat PTSD (Paxil and Zoloft) are antidepressants. These drugs can reduce the general anxiety or comorbid depression associated with PTSD but leave many symptoms untreated (Hetrick, et al., 2010). Alone or combined, these two types of treatment are sometimes effective, but symptoms for many remain refractory (Cooper, Carty, & Creamer, 2005; Hamner & Robert, 2005; Hetrick, et al., 2010). This leaves not only room for improvement in treatment development but also, in the study of the basic psychobiological mechanisms involved in PTSD.

It is important to understand how drugs influence the presentation and formation of PTSD. Of specific interest are opioids. Several clinical studies show a relationship between morphine administration and a reduction in PTSD risk (Nixon, Ellis, Nehmy, & Ball, 2010). In one, the medical records of 696 injured U.S. military personnel without serious traumatic brain injury were analyzed, in cases where morphine was administered during early resuscitation and trauma care, 61% of patients developed PTSD as opposed to 76% of patients who did not receive morphine (Holbrook, Galarneau, Dye, Quinn, & Dougherty, 2010). Results were similar in a sample of 48 adolescents who were examined within 4 weeks of an injury that led to hospital
treatment. Morphine administration was associated with dose dependent reductions in PTSD diagnosis at a 6 month follow-up assessment (Nixon et al., 2010). This result was repeated in a different sample of 90 7-17 year olds assessed in an identical manner (Nixon, Nehmy, et al., 2010; Stoddard et al., 2009) and in an additional sample of 120 trauma victims assessed at 3 months post event (Bryant, Creamer, O'Donnell, Silove, & McFarlane, 2009). These studies show that there is a positive relationship between the dose of morphine administered and a decrease in likelihood of developing PTSD. In sum, these results also show that this relationship exists in humans of diverse ages and trauma sources. It is important therefore to investigate how opioids affect the expression and formation of PTSD. One place to start that large undertaking is to use a preclinical model of the disorder and investigate the effects of opioids on that model.

Posttraumatic Stress Disorder Criteria

Trauma associated anxiety has been discussed in medical literature earlier than the civil war though not known as PTSD, but by colloquialisms like shell-shock, war neurosis and battle fatigue (Newport & Nemeroff, 2000). It was not until the third edition of the APA’s Diagnostic and Statistical Manual of Mental Disorders (DSM) in 1980 that trauma related anxiety syndromes were recognized and named posttraumatic stress disorder (Newport & Nemeroff, 2000). This inclusion in the DSM was controversial. Many of the disorders symptoms overlapped with anxiety and mood disorders and so both the placement and uniqueness of the disorder was questioned (Horowitz, Weiss, & Marmar, 1987; Kinzie & Goetz, 1996). Since its inclusion in the DSM the diagnosis has undergone refinement and we have gained more knowledge about the unique psychobiology of PSTD that firmly cements the disorder as a distinctive diagnosis (Kellner & Yehuda, 1999; Yehuda, 2000, 2001).
The current diagnostic criteria for PTSD specify it as a complex and lasting anxiety response resulting from exposure to extreme trauma. PTSD presents with characteristic symptoms including persistent re-experiencing of the traumatic event, persistent avoidance of stimuli associated with the trauma and numbing of general responsiveness, and persistent symptoms of increased arousal. The symptoms must be present for more than 1 month and the disturbance must cause clinically significant distress or impairment in social, occupational, or other important areas of functioning (DSM-IV™, 2000). A unique and essential feature of PTSD is the development of these characteristic symptoms following exposure to an extreme traumatic stressor. This stressor involves either direct personal experience of an event that involves death, injury, or a threat to the physical integrity of the person; or witnessing an event that involves death, injury, or a threat to the physical integrity of another person; or learning about unexpected or violent death, serious harm, or threat of death or injury experienced by a family member or other close associate (DSM-IV™, 2000). This criterion is unique in the presentation of psychological disease in that it provides an external marked precipitating event in the development of a psychiatric disorder (A criterion). The disorder may be especially severe or long lasting when the stressor is of human design (e.g., torture, rape). The likelihood of developing this disorder may increase as the intensity of and physical proximity to the stressor increase (DSM-IV™, 2000). Other important criterion are divided into three categories: Re-experiencing the event or the physiological reactive state present in the original instance (B criterion), avoidance of thoughts, feelings, actions or any stimuli that might remind them of or be similar to the original instance (C criterion), as well as a change in the overall basal level of anxiety and reactivity to stressful stimuli (D criterion) known as hyper-arousal (DSM-IV™, 2000).
The clinical B Criterion of re-experiencing the event can present in diverse ways. Commonly the person has recurrent and intrusive recollections of the event (Criterion B1) or recurrent distressing dreams during which the event is replayed (Criterion B2). In rare instances, the person experiences dissociative states that last from a few seconds to several hours, or even days, during which components of the event are relived and the person behaves as though experiencing the event at that moment (Criterion B3). Intense psychological distress (Criterion B4) or physiological reactivity (Criterion B5) often occurs when the person is exposed to triggering events that resemble or symbolize an aspect of the traumatic event (e.g. anniversaries of the traumatic event; hot humid weather for combat veterans of the South Pacific or hot dry weather for veterans of Iraq and Afghanistan; entering any parking garage for a woman who was raped in a parking garage) (DSM-IV™, 2000).

The person will make persistent attempts to limit exposure to stimuli associated with the trauma. They will try to avoid thoughts, feelings, or conversations about the traumatic event (Criterion C1) and to avoid activities, situation, or people who arouse recollections of it (Criterion C2). This avoidance of reminders may include amnesia for an important aspect of the traumatic event (Criterion C3). Diminished responsiveness to the external world, referred to as "psychic numbing" or "emotional anesthesia," usually begins soon after the traumatic event. The individual may complain of having markedly diminished interest or participation in previously enjoyed activities (Criterion C4), of feeling detached or estranged from other people (Criterion C5), or of having markedly reduced ability to feel emotions (especially those associated with intimacy, tenderness, and sexuality) (Criterion C6). The individual may have a sense of a foreshortened future (e.g., not expecting to have a career, marriage, children, or a normal life span) (Criterion C7)(DSM-IV™, 2000).
These continual symptoms of anxiety or increased arousal experienced by the person were not present before the trauma. These symptoms may include difficulty falling or staying asleep that may be due to recurrent nightmares during which the traumatic event is relived (Criterion D1), hypervigilance (Criterion D4), and exaggerated startle response (Criterion D5). Some individuals report irritability or outbursts of anger (Criterion D2) or difficulty concentrating or completing tasks (Criterion D3) (DSM-IV™, 2000).

The diagnosis can also include specifiers denoting onset and duration of symptoms. The diagnosis includes the specifier **Acute** when the duration of symptoms is less than 3 months. When symptoms last 3 months or longer then the diagnosis might include the specifier **Chronic**. Finally the specifier **Delayed Onset** indicates that at least 6 months have passed between the traumatic event and the onset of the symptoms (DSM-IV™, 2000). As with many psychiatric diagnoses there is a very complex set of symptoms associated with PTSD. It is important as with other disorders to attempt to understand the physiological processes involved in the initiation and expression of PTSD.

**Neurobiology of PTSD**

The neurobiological changes which occur as a result of exposure to trauma or stress have been studied with increasing interest since PTSD’s inclusion in the DSM. The changes in neurobiology related to PTSD are complex, involving dysregulation of neurotransmitters such as serotonin and norepinephrine, as well as the sympathetic nervous system (fight or flight) and Hypothalamic-Pituitary-Adrenal (HPA) axis (Heim & Nemeroff, 2009). The bulk of neurobiological PTSD research has concentrated on the HPA axis.

**The Hypothalamic-Pituitary-Adrenal axis.** The HPA axis is the major stress response system and exerts its influence through the activation of adrenal glands through neuropeptide release. Upon exposure to stress, neurons in the hypothalamic paraventricular nucleus secrete
corticotropin-releasing factor (CRF) from the median eminence into the hypothalamo-hypophyseal portal circulation, in which the peptide is transported to the anterior pituitary where it stimulates the production and release of adrenocorticotropic hormone (ACTH).

Adrenocorticotropic hormone, in turn, stimulates the release of glucocorticoids from the adrenal cortex. Glucocorticoids affect metabolism, immune function, and the brain, altering physiological functions and behavior in reaction to the stressor. Multiple brain pathways modulate HPA axis activity. The hippocampus and prefrontal cortex (PFC) inhibit the HPA axis, whereas the amygdala and monoaminergic input from the brainstem stimulate the activity of paraventricular nucleus CRF neurons (Heim & Nemeroff, 2009). Glucocorticoids exert negative feedback control of the HPA axis by regulating hippocampal and hypothalamic paraventricular nucleus neurons, as well as ACTH secretion, through binding to glucocorticoid receptors (GR). Sustained glucocorticoid exposure has adverse effects on hippocampal neurons, including reduction in dendritic branching, and loss of dendritic spines (Arborelius, Owens, Plotsky, & Nemeroff, 1999; Fuchs & Gould, 2000; Nestler et al., 2002).

Exposure to acute stressors activates the HPA axis this should result in an increased level of cortisol (a glucocorticoid released after exposure to stress), but paradoxically studies in combat veterans with PTSD revealed low concentrations of cortisol measured in urine or blood, compared to healthy controls (Yehuda, 2006; Yehuda et al., 1990). This counterintuitive finding has been replicated in Holocaust survivors, refugees, and abused persons with PTSD, although findings are not uniformly consistent across studies (Yehuda, 2006; Yehuda, et al., 1990). Other studies have shown similar to normal or even elevated levels of cortisol, differences in type and timing of the psychological trauma, symptom patterns, comorbidity, personality, and genetic dispositions, among other factors, may contribute to this inconsistency (Meewisse, Reitsma, de Vries, Gersons, & Olff, 2007). Studies using low-dose dexamethasone suppression and
metyrapone testing, two pharmacologic agents that alter the availability of stress hormones exerting feedback on the HPA axis, revealed that hypocortisolism in PTSD occurs in the context of increased sensitivity of the HPA axis to negative glucocorticoid feedback (Yehuda, 2006; Yehuda, Yang, Buchsbaum, & Golier, 2006). Findings of increased GR binding and function support the assumption of increased negative feedback sensitivity of the HPA axis in PTSD (Yehuda, 2006). At the central nervous system (CNS) level, increased cerebrospinal fluid concentrations of CRF have been measured in patients with PTSD, both in single lumbar puncture and serial sampling studies (Baker et al., 1999; Bremner, Licinio, et al., 1997). Sustained elevations in CRF concentrations were observed despite comparably low cortisol concentrations, and the latter were negatively correlated with PTSD symptoms (Baker, et al., 1999). There is evidence of blunted adrenocorticotropic hormone response to intravenous cortisol stimulation in PTSD patients (Yehuda, 2006). The below normal levels of adrenocorticotropic hormone in response to exogenous cortisol in PTSD patients when compared to healthy volunteers suggests that the glucocorticoid system is less active in PTSD patients possibly due to a reduction in the total response levels of the system. One mechanism that may explain this is the downregulation of CRF receptors as a compensatory response to elevated cortisol levels seen in early diagnosed PTSD patients (Yehuda, 2006). In addition, reduced volume of the hippocampus, the major brain region inhibiting the HPA axis, is a cardinal feature of PTSD (Bremner, Elzinga, Schmahl, & Vermetten, 2008). Taken together, the specific constellation of neuroendocrine findings in PTSD reflects sensitization of the HPA axis to exposure to stressors. This neuroendocrine pattern distinguishes PTSD from major depression, a frequently comorbid but distinct disorder (Yehuda, 2006).
Interestingly, prospective studies have shown that low cortisol levels at the time of exposure to psychological trauma predict the development of PTSD (Resnick, Yehuda, Pitman, & Foy, 1995; Yehuda, McFarlane, & Shalev, 1998), suggesting that hypocortisolism might be a preexisting risk factor that is associated with maladaptive stress responses such as PTSD. Consequently, administration of hydrocortisone directly after exposure to psychological trauma has been shown to reduce the risk for later development of PTSD in humans in several studies (de Quervain, 2008; Schelling et al., 2004). In addition, it was recently demonstrated that hydrocortisone treatment, simulating normal circadian cortisol rhythm, is effective in reducing some symptoms PTSD (Aerni et al., 2004). Indeed, decreased availability of cortisol, and hence lack of regulatory effects in the CNS, may have permissive effects towards the sustained activation of neural systems involved in stress reactivity and fear processing, including the CRF and norepinephrine (NE) systems (Heim, Ehlert, & Hellhammer, 2000; Yehuda, 2006). Glucocorticoids further interfere with the retrieval of traumatic memories and thereby may prevent or reduce symptoms of PTSD (de Quervain, 2008; de Quervain & Margraf, 2008).

**Hypothalamic-Pituitary-Thyroid Axis.** The HPT axis is an additional response to stress that has only more recently been studied in relation to PTSD. When the HPT axis is activated thyrotropin-releasing hormone (TRH) is released from the hypothalamus which stimulates the secretion of thyrotropin stimulating hormone (TSH) from the anterior pituitary gland. TSH, in turn, stimulates the thyroid gland to secrete thyroxine (T4) and tri-iodothyronine (T3). The thyroid axis is capable of emergency responses and increased T4 levels are part of an arousal signal (Mason, 1968). Increased HPT axis activity has been observed in PTSD patients as well as a link between hyperthyroidism and traumatic stress (Prange, 1999). Specifically the peripheral measures of the total and free fractions of T3 and T4 are elevated in patients with PTSD (Newport
& Nemeroff, 2000). The relative increases of T₃ and T₄ are disproportionate with a significantly higher increase occurring in T₃ levels than T₄ levels. This suggests an increase in the peripheral de-iodination of T₄ to the more biologically active T₃. This supports the observation of a sensitized response of the HPT system in PTSD patients. Which is further maintained by the elevated TSH release that occurs in PTSD patients when TRH is administered under testing conditions (Prange, 1999).

**Hippocampus.** The most reproducible finding in structural imaging studies of PTSD is reduced volume of the hippocampus. The hippocampus is implicated in the control of stress responses, declarative memory and contextual aspects of fear conditioning, and is known as one of the most plastic regions in the brain. As noted above, prolonged exposure to stress and high glucocorticoid levels damages the hippocampus, leading to reduction in dendritic branching, loss of dendritic spines, and impairment of neurogenesis (Fuchs & Gould, 2000). Initial magnetic resonance imaging studies demonstrated smaller hippocampal volumes in Vietnam veterans with PTSD and patients with abuse-related PTSD compared to controls (Bremner et al., 1995; Bremner, Randall, et al., 1997; Gurvits et al., 1996; Stein, Koverola, Hanna, Torchia, & McClarty, 1997). Small hippocampal volumes were associated with the severity of trauma and memory impairments in these studies. These findings were generally replicated in subsequent studies. Studies using proton magnetic resonance spectroscopy (MRS) observed reduced levels of N-acetyl aspartate (NAA), a marker of neuronal integrity, in the hippocampus of adult patients with PTSD (Rauch, Shin, & Phelps, 2006). Of note, NAA reductions were correlated with serum cortisol concentrations (Neylan et al., 2003). Interestingly, reduced hippocampal volume was not observed in children with PTSD (De Bellis et al., 1999). Hippocampal volume reduction in PTSD may reflect toxic effects over time of repeatedly increased glucocorticoid exposure or
increased glucocorticoid sensitivity, though recent evidence also suggests that a small hippocampus might represent a preexisting vulnerability factor for developing PTSD (Pitman et al., 2006). Moreover, in patients with major depression an early life trauma in the form of childhood abuse is associated with reduced hippocampal volume (Vythilingam et al., 2002). Indeed, hippocampal deficits may promote activation of and failure to shut down stress responses, and may contribute to impaired extinction of conditioned fear as well as deficits in discriminating between safe and unsafe contexts. Studies using functional neuroimaging have further revealed that PTSD patients exhibit deficits in hippocampal activation during a verbal declarative memory task (Bremner et al., 1999). Both hippocampal atrophy and functional deficits reverse after successful treatment with selective serotonin reuptake inhibitors (commonly used to treat depression) (Bremner & Vermetten, 2004), which have been demonstrated to increase neurotrophic factors and neurogenesis in preclinical studies (Nestler, et al., 2002).

**Amygdala.** In addition to the hippocampus, other brain structures implicated in a neural circuitry of stress include the amygdala and the prefrontal cortex. The amygdala is a critical limbic structure involved in emotional processing and in the acquisition of fear responses. The amygdala is connected to both cortical and subcortical regions. The basolateral complex is innervated by neocortical and subcortical sensory regions and sends information to the central nucleus of the amygdala. The central nucleus projects to the midbrain and brainstem nuclei to coordinate rapid autonomic, endocrine, and behavioral responses to danger. The central nucleus also receives visceral information from brainstem regions. Connections between the amygdala and the hippocampus are implicated in context conditioning. Connections between the PFC and the amygdala modulate stress responsiveness and mediate extinction of fear memory, inasmuch as the PFC exerts inhibitory control over the amygdala (Schulkin, 2006). The functional role of
the amygdala in mediating both stress responses and emotional learning suggests that changes in this region and its connected circuitry may be implicated in the pathophysiology of PTSD. Although there is no clear evidence for structural alterations of the amygdala in PTSD, functional imaging studies have revealed hyperresponsivity of the amygdala in PTSD during the presentation of traumatic scripts, cues, and other reminders (Liberzon & Sripada, 2008; Shin, Rauch, & Pitman, 2006). PTSD patients further show increased amygdala responses to general emotional stimuli that are not associated with the trauma, such as emotional faces (Shin, et al., 2006). Of note, the amygdala also seems to be sensitized to subliminally presented threatening cues in PTSD (Hendler et al., 2003; Rauch et al., 2000; Schulkin, 2006). Increased amygdala activation has also been reported for PTSD patients during fear acquisition in a conditioning experiment (Bremner et al., 2005). Given that increased amygdala reactivity has been linked to genetic traits (Hariri et al., 2002), which moderate risk for PTSD (Kilpatrick et al., 2007), increased amygdala reactivity may represent a biological risk factor for the development of PTSD.

**Prefrontal Cortex.** The medial prefrontal cortex (mPFC) comprises the anterior cingulate cortex (ACC), subcallosal cortex, and the medial frontal gyrus. The mPFC is connected with the amygdala, where it exerts inhibitory control over stress responses and emotional reactivity. The mPFC further mediates extinction of conditioned fear through active inhibition of acquired fear responses (Shin, et al., 2006). Patients with PTSD exhibit decreased volumes of the frontal cortex (Rauch et al., 2003), including reduced volumes of the ACC (Woodward et al., 2006; Yamasue et al., 2003). The reduction in ACC volume was correlated with PTSD symptom severity in some of these studies. Altered shape of the ACC (Corbo, Clement, Armony, Pruessner, & Brunet, 2005) and decreased NAA concentrations in the ACC (De Bellis,
Keshavan, Spencer, & Hall, 2000) have also been reported in PTSD patients. A recent twin study suggests that volume loss in the ACC is an acquired correlate of having PTSD, rather than a preexisting risk factor (Kasai et al., 2008). Functional imaging studies have found decreased activation of the mPFC in PTSD patients in response to stimuli, such as traumatic scripts (Britton, Phan, Taylor, Fig, & Liberzon, 2005; Shin et al., 2004), combat pictures and sounds (Bremner, et al., 1999), trauma unrelated negative narratives (Lanius et al., 2003), fearful faces (Shin et al., 2005), emotional Stroop(Bremner et al., 2004), and others, though there are also discordant findings (Shin, et al., 2006). Reduced activation of the mPFC was associated with PTSD symptom severity in several of these studies and successful selective serotonin re-uptake inhibitor (SSRI) treatment restored mPFC activation patterns(Shin, et al., 2006). Of note, in the above cited conditioning experiment (Bremner, et al., 2005), extinction of conditioned fear was associated with decreased activation of the ACC, providing a biological basis for imprinted traumatic memories in PTSD. Given the connectivity between the amygdala and mPFC, interactions in activation patterns between these regions have been reported in PTSD, though the direction of the relationship is inconsistent across studies (Shin, et al., 2006).

**Catecholamines.** The catecholamines comprise a family of neurotransmitters derived from the amino acid tyrosine. The rate-limiting factor in the synthesis of catecholamines is tyrosine hydroxylase, an enzyme that converts tyrosine into DOPA, which subsequently is converted into dopamine (DA) by the action of DOPA decarboxylase. In noradrenergic neurons, dopamine β hydroxylase converts DA into NE. NE is one of the principal mediators of the CNS and autonomic stress responses. The majority of CNS NE is derived from neurons of the locus ceruleus (LC) that project to various brain regions involved in the stress response, including the PFC, amygdala, hippocampus, hypothalamus, periaqueductal grey, and thalamus. There is
evidence for a feed-forward circuit connecting the amygdala and the hypothalamus with the LC, in which CRF and NE interact to increase fear conditioning and encoding of emotional memories, enhance arousal and vigilance, and integrate endocrine and autonomic responses to stress. Glucocorticoids inhibit this cascade (Pavcovich & Valentino, 1997). In the periphery, sympathoadrenal activation during exposure to stressors results in the release of NE and epinephrine from the adrenal medulla, increased release of NE from sympathetic nerve endings, and changes in blood flow to a variety of organs, reflecting an alarm reaction that mobilizes the body to allow for optimal coping. The effects of NE are mediated via postsynaptic $\alpha_1$, $\beta_1$ and $\beta_2$ receptors, whereas another NE-activated receptor, the $\alpha_2$ receptor, serves as a presynaptic autoreceptor inhibiting NE release (Koob, 1999). Because of its multiple roles in regulating arousal and autonomic stress responses, as well as promoting the encoding of emotional memories, NE has been a central candidate in studying the pathophysiology of PTSD.

A cardinal feature of patients with PTSD is sustained hyperactivity of the sympathetic branch of the autonomic nervous system, as evidenced by heart rate, blood pressure, skin conductance level, and other psychophysiological measures. Accordingly, increased urinary excretion of NE and epinephrine, and their metabolites, has been documented in combat veterans, abused women, and children with PTSD. In addition, patients with PTSD exhibit increased heart rate, blood pressure, and NE responses to challenge, such as traumatic reminders. Decreased platelet $\alpha_2$ receptor binding further suggests NE hyperactivity in PTSD (Strawn & Geracioti, 2008; Vermetten & Bremner, 2002). There is also evidence for a role of altered CNS NE function in PTSD. Administration of the $\alpha_2$ receptor antagonist yohimbine, which increases NE release, induces symptoms of flashbacks and increased autonomic responses in patients with PTSD (Southwick et al., 1999). Serial sampling revealed sustained increases in CSF NE
concentrations and increased CSF NE responses to psychological stressors in PTSD (Geracioti et al., 2001; Geracioti et al., 2008). Taken together, increased CNS NE activity plausibly contributes to features of PTSD, including hyperarousal, increased startle, and encoded fear memories (Strawn & Geracioti, 2008).

Interestingly, prospective studies have shown that increased heart rate and peripheral epinephrine excretion at the time of exposure to trauma predict subsequent development of PTSD (Delahanty & Nugent, 2006; Yehuda, et al., 1998). Remarkably, administration of the centrally acting β adrenergic blocker propranolol shortly after exposure to psychological trauma has been reported to reduce PTSD symptom severity and reactivity to reminders of the traumatic event (Pitman et al., 2002). Although this did not prevent the development of PTSD, it may have blocked traumatic memory consolidation (Brunet et al., 2008), and therefore may reduce the severity or chronicity of PTSD. Various anti-adrenergic agents have been tested for their therapeutic efficiency in the treatment of PTSD in open label trials, though there is a paucity of controlled trials (Strawn & Geracioti, 2008).

It should be noted that increased urinary excretion of DA and its metabolite has been reported for patients with PTSD. At the CNS level, mesolimbic DA plays a critical role in the processing of rewards. DA has also been implicated in fear conditioning. There is evidence in humans that exposure to stressors induces mesolimbic DA release, which in turn may impact on HPA axis responses. Whether or not the CNS DA system is altered in PTSD remains unclear, though genetic variations in the DA system have been implicated in moderating risk for PTSD.

**Serotonin.** Serotonin, also known as 5-hydroxytryptamine (5-HT), is a monoamine neurotransmitter synthesized from the amino acid tryptophan. Serotonergic neurons originate in the dorsal and medial nuclei raphé in the brainstem and project to multiple forebrain regions,
including the amygdala, bed nucleus of the stria terminalis, hippocampus, and PFC. This indoleamine has roles in regulating sleep, appetite, sexual behavior, aggression/impulsivity, motor function, analgesia, and neuroendocrine control. It also has been implicated in the pathophysiology of mood and anxiety disorders and in the modulation of affective and stress responses. The direction of the modulatory effects of 5-HT on affective and stress responses depends on stressor intensity, brain region, and receptor type. It is believed that 5-HT neurons of the dorsal raphe projecting to the amygdala and hippocampus mediate anxiogenic (stress-increasing) effects via 5-HT2 receptors, whereas 5-HT neurons from the median raphe exert anxiolytic effects, facilitate extinction, and suppress encoding of learned associations via 5-HT1A receptors. Chronic exposure to stressors induces upregulation of 5-HT2 and downregulation of 5-HT1A receptors, respectively, in animal models. 5-HT1A receptor knockout mice exhibit increased stress responses. The 5-HT system interacts with the CRF and NE systems in coordinating affective and stress responses (Ressler & Nemeroff, 2000; Vermetten & Bremner, 2002). Indirect evidence suggests a role of 5-HT in the pathophysiology of PTSD, including symptoms of impulsivity, hostility, aggression, depression, and suicidality. Most important concerning a role of 5-HT circuits in PTSD is the demonstrated partial efficacy of the SSRIs as treatments. However, though their use is indeed recommended in many current treatment guidelines the Institute of Medicine concluded that there is insufficient evidence for the efficacy of the approved medications for PTSD, Sertraline and Paroxetine (Institute of Medicine (U.S.). Committee on Treatment of Posttraumatic Stress Disorder., 2008).

Other evidence for altered 5-HT neurotransmission in PTSD includes decreased serum concentrations of 5-HT, decreased density of platelet 5-HT uptake sites, and altered responsiveness to CNS serotonergic challenge (Ressler & Nemeroff, 2000; Vermetten & Bremner, 2002). However, no differences in CNS 5-HT1A receptor binding were detected in
patients with PTSD compared to controls using positron emission tomography (PET) imaging (Bonne et al., 2005). Taken together, altered 5-HT transmission may contribute to symptoms of PTSD such as hypervigilance, increased startle, impulsivity, and intrusive memories.

**GABA/Benzodiazepine Receptor System.** Gamma-aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the CNS. GABA exerts anxiolytic effects and dampens behavioral and physiological responses to stressors, in part by inhibiting the CRF/NE circuits involved in mediating fear and stress responses. GABA acts on GABAA receptors, part of the GABAA/benzodiazepine (BZ) receptor complex. Uncontrollable stress has been shown to lead to alterations in the GABAA/BZ receptor complex. Treatment with BZ, GABA agonists, or GABA reuptake inhibitors decreases symptoms of anxiety in PTSD, suggesting that the GABA/BZ system may be involved in the pathophysiology of PTSD. Patients with PTSD exhibit decreased platelet BZ binding sites (Gavish et al., 1996). Single photon emission computed tomography and PET imaging studies revealed decreased BZ receptor binding in the cortex, hippocampus and thalamus of patients with PTSD. These results suggest that decreased density or affinity of the BZ receptor may play a role in PTSD (Bremner et al., 2000; Geuze et al., 2008). However, treatment with BZs after exposure to psychological trauma does not prevent PTSD (Gelpin, Bonne, Peri, Brandes, & Shalev, 1996). Although there are multiple studies implicating the GABA/BZ receptor system in anxiety disorders, studies in PTSD are relatively sparse (Strawn & Geracioti, 2008).

**Glutamate/NMDA Receptor System.** Glutamate is the primary excitatory neurotransmitter in the CNS. Exposure to stressors and the release or administration of glucocorticoids increases glutamate release in the brain. Glutamate binds to several so-called excitatory amino acid receptors, one of which is the N-methyl-D-aspartate (NMDA) receptor.
The glutamate/NMDA receptor system has been implicated in synaptic plasticity, learning, and memory, including the well studied phenomenon of long term potentiation (LTP), the extended excitation of neural circuits, leading to long-lasting enhancement in communication between neurons. This process is believed in part to underlie the process of conditioning and memory consolidation. LTP plausibly contributes to consolidation of trauma memories in PTSD. Of note, the partial NMDA-receptor antagonist D-cycloserine has been shown to improve the extinction of fear in rodents and in phobic patients undergoing exposure therapy. D-cycloserine has been shown in many studies to enhance learning of many types including extinction learning (Choi, Rothbaum, Gerardi, & Ressler, 2010; Kaplan & Moore, 2011). Whether or not D-cycloserine is effective in enhancing the outcome of exposure therapy in PTSD remains to be studied (Davis, Ressler, Rothbaum, & Richardson, 2006). In addition to its role in learning and memory, overexposure to glutamate is associated with excitotoxicity, and plausibly could contribute to a loss of neurons in the hippocampus and PFC in PTSD. Of note, elevated glucocorticoids increase the expression and/or sensitivity of NMDA receptors, which may sensitize the brain to excitotoxic insults.

Neuropeptide Y. Neuropeptide Y (NPY) is a neuropeptide with anxiolytic and stress-buffering properties. NPY has been shown to inhibit CRF/NE circuits involved in stress and fear responses and reduces the release of NE from sympathetic nerve cells. A relative lack of NPY may promote maladaptive stress responses and contribute to the development of PTSD. Indeed, patients with PTSD have been reported to exhibit decreased plasma NPY concentrations and blunted NPY responses to yohimbine challenge compared to controls, suggesting that decreased NPY activity may contribute to noradrenergic hyperactivity in PTSD (Rasmusson et al., 2000). However, it has been suggested that NPY may be involved in promoting recovery from or
resilience to PTSD because combat veterans without PTSD have been demonstrated to exhibit elevated plasma NPY levels compared to veterans with PTSD (Yehuda, Brand, & Yang, 2006).

**Opioids.** Endogenous opioids, such as the endorphins, enkephalins and dynorphins are endogenous neuropeptides that act upon opiate receptors (which can also be activated by synthetic or naturally occurring opiates like morphine or heroin). Endorphins work as an agonist at and have a high affinity for mu receptors but also act at and have a slightly lower affinity for kappa receptors. β-Endorphin is a cleavage product of pro-opiomelanocortin (POMC), which is also the precursor hormone for adrenocorticotropic hormone. Dynorphins act primarily through the kappa receptor but can have some activity through mu receptors. Enkephalins are divided into leu-enkephalin which work through delta type opioid receptors and met-enkephalin which works through mu and delta receptors. Alterations in endogenous opioids have been postulated to be involved in symptoms of numbing, stress-induced analgesia, and dissociation in PTSD (Heim & Nemeroff, 2009). Endogenous opioids further exert inhibitory influences on the HPA axis as evidenced by attenuated stress induced release of NE in the thalamus, hypothalamus, hippocampus, amygdala and midbrain when rats were pre-treated with morphine (Tanaka et al., 1983). Opiate effects may occur through a reduction in the firing of the LC and may explain heroin’s alleviation of the hyperarousal symptoms of some PTSD patients (Bremner, Southwick, Darnell, & Charney, 1996). Heroin addicted individuals in comparison with healthy volunteers have been shown to exhibit significantly lower levels of adrenocorticotropic hormone, as well as have reduced levels HPA axis activation in response to a stressor (Gerra et al., 2004; Ho et al., 1977). Naloxone, an opiate receptor antagonist, increases HPA axis activity by blocking an inhibitory opiodergic influence on hypothalamic CRF secretion, and patients with PTSD have been reported to exhibit an exaggerated HPA axis response to naloxone. Interestingly, naloxone
also has been shown to reverse the analgesia of PTSD patients after exposure to traumatic reminders. Also, PTSD patients exhibit increased CSF β-endorphin levels, suggesting increased activation of the endogenous opioid system. Interestingly, the opiate receptor antagonist, naltrexone, has been reported to be effective in treating symptoms of dissociation and flashbacks in traumatized patients (Newport & Nemeroff, 2000; Strawn & Geracioti, 2008). Finally, administration of morphine has been linked to a reduction in risk of the development of PTSD (Bryant, et al., 2009; Holbrook, et al., 2010; Nixon, Nehmy, et al., 2010; Stoddard, et al., 2009).

Several clinical studies show a relationship between morphine administration and a reduction in PTSD risk (Nixon, Ellis, et al., 2010). In one, the medical records of 696 injured U.S. military personnel without serious traumatic brain injury were analyzed, in cases where morphine was administered during early resuscitation and trauma care, 61% of patients developed PTSD as opposed to 76% of patients who did not receive morphine (Holbrook, et al., 2010). Results were similar in a sample of 48 adolescents who were examined within 4 weeks of an injury that led to hospital treatment. Morphine administration was associated with dose dependent reductions in PTSD diagnosis at a 6 month follow-up assessment (Nixon, Nehmy, et al., 2010). This result was repeated in a different sample of 90 7-17 year olds assessed in an identical manner (Nixon, Nehmy, et al., 2010; Stoddard, et al., 2009) and in an additional sample of 120 trauma victims assessed at 3 months post event (Bryant, et al., 2009). These studies show that there is a positive relationship between the dose of morphine administered and a decrease in likelihood of developing PTSD. In sum, these results also show that this relationship exists in humans of diverse ages and trauma sources. There are at least three possible mechanisms by which opioids may protect against PTSD; reducing pain, preventing memory consolidation, and modulation of HPA axis activity.
Opioids exert pain relief through the activation of specific membrane receptors. There are three major subtypes of receptors, mu, kappa, and delta, which are located in diverse areas of the central nervous system (Kanjhan, 1995). The brain contains multiple endogenous opioid peptides: enkephalins, dynorphins, and endorphins. These peptides are released into the brain and blood following stress or pain (Akil et al., 1984). Both endogenous opioid peptides mentioned above and exogenous opioid peptides (produced outside the body either botanically or chemically) can relieve pain. Some literature suggests that pain relief may be the primary mechanism by which morphine may reduce PTSD risk. The relief of pain may make the trauma less stressful (Stoddard, et al., 2009).

Opioids have been repeatedly shown to affect and regulate memory. This is accomplished physiologically by beta-endorphin’s release after exposure to a novel situation. This state dependent effect is reversible by mu antagonists like naloxone. This occurs in several brain areas including the amygdala. In addition to this state dependant effect, administration of mu opioid agonists reduces memory retention, which may be due to an amnesiac effect (Izquierdo et al., 1980). For example, in healthy human volunteers subtle working memory impairments were found in women following both oxycodone and morphine administration (Friswell et al., 2008). In a preclinical model, a pre-training single administration of morphine has been observed to decrease the spatial memory acquisition in Morris water maze task in rats (Farahmandfar, Karimian, Naghd, Zarrindast, & Kadivar, 2010). Therefore, morphine may have memory effects that prevent conditioning of traumatic stimuli.

The kappa opioid receptor (KOR) system has also been highly characterized with regards to stress. The data supports that the kappa system influences and can be influenced by stress. A variety of studies and reviews support endogenous opioid peptide systems involvement in the mediation, modulation, and regulation of stress responses. Specifically the kappa opioid receptor
The widespread distribution of enkephalin throughout the limbic system is consistent with the kappa receptor system playing a direct role in the modulation of the stress response (Drolet et al., 2001). Kappa opioid receptor antagonists have been found to block the aversive behaviors brought on by forced swim (Beardsley, Howard, Shelton, & Carroll, 2005) and inescapable footshock stress (Land et al., 2008). Opioid analgesics, though mostly active at mu receptor subtypes, often act at other receptor subtypes like kappa. Thus it is important to investigate kappa compounds as well as analgesics like morphine to help us understand if opioid stress interactions are a part of morphine’s protective effect.

**Psychological Approaches to the Treatment of PTSD**

There are a large variety of psychological approaches to treating PTSD that may be used singly or in conjunction. The most widely used of these treatments are based on the concepts and traditions of cognitive behavioral therapy (Sharpless & Barber, 2011). Pharmacotherapy approaches mentioned previously are often explored in addition to these psychological therapies.

**Prolonged exposure.** The foundations of prolonged exposure are closely related to extinction learning and are intended to modify the memory processes first changed in the traumatic exposure. The treatment usually consists of 8-15 weekly 90-minute sessions. There are three main components; visualization and examination of traumatic memories, discussion and examination of these memories along with *in vivo* exposure to trauma related stimuli and situations in a safe controlled environment (Sharpless & Barber, 2011). The prolonged exposure treatment method has the most data supporting its treatment efficacy as well as being one of two treatments currently used by the Veterans Administration (VA) (Powers, Halpern, Ferenschak, Gillihan, & Foa, 2010). Exposure therapy can also be modeled in preclinical assays and is more
effective when given in close temporal proximity to the initiating event. Mice, which were given extinction therapy 24h after and one month after Pavlovian conditioning, had reduced freezing behavior relative to controls, but only those given therapy at the 24h after time point showed a reduction in hyperarousal symptoms (Golub, Mauch, Dahlhoff, & Wotjak, 2009).

**Cognitive processing therapy.** Though some components of cognitive processing therapy have similarities to cognitive behavioral therapy as well as prolonged exposure the treatment focuses on self-blame (Resick & Schnicke, 1992). The exposure component of cognitive processing therapy is written rather than mental imagery. Specifically, clients are instructed to *write* about their traumatic events in detail, review them daily and share them aloud during sessions. The clients are assisted in labeling feelings and working through “stuck points” in the narratives. The focus of this review is to process individual components of the experience and how each makes them feel and think about the events (Sharpless & Barber, 2011). Cognitive processing therapy has very good data supporting its use in PTSD (Forbes et al., 2012), and it was chosen as the other psychological treatment to be extensively “rolled out” through the VA system.

**Eye movement desensitization and reprocessing.** This treatment combines elements of cognitive behavioral therapy, mindfulness, body-based approaches, and person-centered therapies. It is clinically guided by the Adaptive Information Processing Model (Shapiro & Maxfield, 2002) that proposes that traumatic memories in PTSD are unprocessed and are not stored as memories, but are treated as if they were new sensory inputs. There are eight phases of treatment in eye movement desensitization and reprocessing, of which the most unique are termed desensitization and reprocessing (when clients hold distressing images in mind while tracking rhythmic finger movements of the clinician), the installation of positive cognitions
(during which fingers are tracked while holding positive cognitions in mind), and journaling (Shapiro & Maxfield, 2002).

**Stress inoculation training.** Initially developed to manage anxious symptoms the group of techniques known as stress inoculation training (relaxation, thought stopping, *in vivo* exposure to feared situations) has been subsequently adapted to PTSD and other specific disorders (E. B. Foa, Rothbaum, Riggs, & Murdock, 1991). Stress inoculation training has been shown to be moderately effective in reducing PTSD symptoms, though eye movement desensitization and reprocessing is slightly more effective (Lee, Gavriel, Drummond, Richards, & Greenwald, 2002). More study should be done to discover which components are key to its success due to inconsistent data (Edna B. Foa & International Society for Traumatic Stress Studies., 2009).

**Exposure therapy using virtual reality.** With the advancements in technology we now have the ability to use virtual exposure to stimuli instead of imagined or *in vivo*. Exposure therapy using virtual reality may include convincing visual stimuli, 3D sound, smells, and a general feeling of immersion in traumatic situations (Rothbaum, Rizzo, & Difede, 2010). This treatment has been used with Iraq veterans in 19 military centers, and has seen some modest success, especially with veterans who have difficulty with visualizing trauma or talk therapy (Rothbaum, et al., 2010).

**Relaxation training.** Relaxation training may have been the earliest behavioral treatment used for PTSD, and consists of using various techniques (e.g., successive tension and relaxation of muscles) in order to reduce the fear and anxiety associated with traumatic responses. It has been used as a standalone treatment (often as a control) and as a component of broader PTSD treatments. Relaxation training has been used in four randomized clinical trials, and while
certainly effective, it is not as effective as more comprehensive treatment packages (Sharpless & Barber, 2011).

**Cognitive behavioral group therapy.** This treatment is as its name suggests cognitive behavioral therapy in a group setting. While this treatment approach has been shown to be effective, it has not been shown to be significantly better than other nonspecific treatment control groups (Schnurr et al., 2003).

In summary, of the psychological therapies described here (i.e., those which have undergone the most empirical testing), prolonged exposure, cognitive processing theory, and eye movement and desensitization and reprocessing possess the most evidence in favor of their efficacy and utility with veterans (Sharpless & Barber, 2011). However, these therapies are not effective in many cases and many patients are still refractory (Hetrick, et al., 2010). As described previously pharmacotherapies are also not effective in a large number of cases. This leaves considerable room for improvement in treatment development. It is important to understand how different drug treatments like opiates affect fear behavior so that better treatments or combination therapy approaches can be developed.

**Preclinical Models of PTSD**

While the complexity of psychiatric disorders like PTSD cannot be fully replicated in a preclinical assay, there are many existing preclinical assays that can be used to model key parts of the disease. Some of these preclinical models observe and record well explored behaviors in animals and how those behaviors might change in relation to stress. Other preclinical PTSD models are newer adaptations of existing assays like fear potentiated startle, which combines an associative learning component with a startle reflex two components now understood to be a part of PTSD’s disease mechanism. There are different approaches to creating a preclinical PTSD model (i.e. stress/trauma initiated, mechanism based, neurobiological system and genetic
There are benefits and limitations on each approach, it is important to understand the approaches and how they impact the conclusions that can be drawn from the resulting data.

**Neurobiological systems models of PTSD.** Neurobiological models seek to initiate biological systems changes (including the HPA axis) that mimic those that occur in PTSD patients. Changes in neuroendocrine function and arousal that are characteristic of PTSD can be induced by single prolonged stress exposure in rats. Decreased neural activity in the prefrontal cortex, increased neural activity in the amygdala complex, and reduced neuronal integrity in the hippocampus is associated with PTSD (Knox, Perrine, George, Galloway, & Liberzon, 2010). The single prolonged stress exposure models recreate to some extent these aspects of PTSD.

**Genetic model approaches to PTSD.** The availability of the fully sequenced mouse genome and the tools to manipulate that genome (gene knock-out, transgenic and gene silencing) give us great tools to approach genetic influences in many disease states. The difficulty with PTSD and many other psychiatric disorders is that there is no single gene or small group of genes wholly responsible for the disorder. The picture that seems to be arising both from clinical and preclinical studies is that there are genes which give rise to a certain predisposition for developing a disorder given the addition of a certain environmental events. As described above, there are many systems involved in the body’s complex stress response. Therefore, many genetic and likely epigenetic factors are involved in the vulnerability for developing PTSD. At this point in time, distinct genetic models do not exist for PTSD (Schmidt, Holsboer, & Rein, 2011).

**Trauma/Stress initiated models of PTSD.** Trauma/Stress initiated models of PTSD. These assays seek to employ an initiating stress or trauma to represent the criterion A (direct personal experience of an event that involves death, injury, or a threat to the physical integrity)
of PTSD. This approach can include a physical stressor or a psychosocial stressor like predator exposure. In addition, ethologically relevant stressors, such as predator exposure, produce lasting increases in stress-related behavior and levels of corticosterone in plasma. Reports also indicate habituation is less likely to occur with repeated exposure to a predator than with repeated exposure to different stressors such as restraint (Plata-Salaman et al., 2000). While these models are functional and provide data at the behavioral level, many do not shed light on underlying brain mechanisms that are responsible for producing this behavior.

**Mechanism based models of PTSD.** The trauma or stress initiated models do a good job of modeling short term stress behavior, but do not translate completely to the persistence and resistance of symptoms present in the disorder. The fear conditioning paradigm mirrors the learning and memory processes that occur in humans during traumatic exposure and displays many signs that can be directly correlated to those seen in PTSD (E. B. Foa & Kozak, 1986; Maren, 2001). In the mouse, Pavlovian fear conditioning (PFC) is observed by the animal freezing in place. This freezing behavior is a natural response to threat (Cantor, 2009). A mouse is put into a test chamber and exposed to an aversive stimulus (i.e. shock) that is paired with a neutral stimulus (i.e. white noise) and the mouse then associates the light with the shock. During Pavlovian fear conditioning this association between an aversive stimulus and accompanying neutral stimuli is formed via activation of various brain areas including the hippocampus and amygdala (Kim & Jung, 2006; Maren, 2008). The associative memory processes activated during this type of conditioning have also been observed in humans through imaging studies in PTSD patients (Bremner, 2004; Bremner, et al., 2005).

Multiple symptoms, both behavioral and biological, found in PTSD patients are modeled by PFC. Mice that are exposed to PFC show hippocampal volume changes that are similar to PTSD patients. After undergoing an inescapable footshock reduced hippocampal volume is
observed in mice (Golub et al., 2011). PTSD patients also show reduced hippocampal volume (Bremner, et al., 1995; Bremner, Randall, et al., 1997; Gurvits, et al., 1996; Stein, et al., 1997). Hyperarousal is another symptom observed in this preclinical model, as well as, clinical PTSD patients (Harrington et al., 2012). Mice display hyperarousal after PFC (Golub, et al., 2009) this hyperarousal is sensitive to extinction training. Exaggerated startle response is observed in PTSD patients (Asmundson & Carleton, 2010). PFC results in a similar increased response and the study of this specific response uses PFC prior to startle assays and is now known a the fear potentiated startle assay (Smith et al., 2010). Symptomatology in PTSD is long lasting, with some patients having unresolved symptoms decades after their initial diagnosis (Bremner, et al., 1996; Malta, Wyka, Giosan, Jayasinghe, & Difede, 2009). The changes in behavior and brain structures persist for longer than 4 weeks in this preclinical model (Li, Murakami, Wang, Maeda, & Matsumoto, 2006).

The behavioral and biological similarity to posttraumatic stress disorder displayed by this particular assay, Pavlovian Fear Conditioning, makes it especially attractive to use when investigating specific pharmacological interventions. The ability to manipulate through pharmacological intervention or conditioning to fear responses or extinction make the type of data that can be generated by this assay especially diverse and informative in the context of a specific receptor system. The range of symptoms represented, in addition to the biological similarities of PFC to PTSD, provide a strong preclinical model that would more than likely be able to detect candidate compounds for the treatment of PTSD.
Rationale

Post Traumatic Stress Disorder (PTSD) is an anxiety disorder that affects over 7.7 million adults and carries an estimated societal cost of $6.2 billion. This prevalence and therefore the cost of PTSD is increasing. Current treatments for PTSD include psychotherapy (e.g., exposure therapy) and pharmacotherapy (e.g., antidepressants). Although there are a wide variety of therapies that have been used and new ones are being developed, there is still no clear treatment approach that does not leave a large portion of patients still suffering or that have spontaneous reoccurrences of symptoms.

Our increased knowledge about PTSD has revealed that there is dysregulation of many neurotransmitter systems in patients diagnosed with the disorder. One of the systems that is affected is the endogenous opioid system. In addition independent analyses of medical records indicate that exogenous opiate administration (morphine) may have a protective affect against PTSD. Opioid analgesics, including morphine and fentanyl, are often administered as a response to trauma. Since opioids, both endogenous and exogenous, influence neurological processes that we know are affected in those with PTSD it is important to study how these compounds exert this effect. This is a novel PTSD treatment avenue that has not yet been explored.

Preclinical models of psychiatric disorders are established tools that can be used to reveal mechanisms, etiology, treatments and many other important factors of these disorders. Pavlovian fear conditioning is believed to model the memory processes that take place in PTSD development and has clear parallels to many of its symptoms. The open field assay can be a measure of both anxiety and avoidance behaviors while serving as a control to indicate if tested compounds have sedative or stimulant effects not related to fear.
I propose using the preclinical assays Pavlovian fear conditioning and the open field to illuminate the effects of opioids on fear behavior that models PTSD.

Because of the clinical observations involving morphine administration and PTSD;

I hypothesize that opioid compounds with mu opiate receptor (MOR) agonist properties will decrease fear behavior in both open field and Pavlovian fear conditioning.

Also, because of previous research involving KOR antagonists;

I hypothesize that compounds with KOR antagonist properties will decrease fear behavior in both the open field and Pavlovian fear conditioning.
Methods Experiment I

The following is the first of two experiment methods; this project is broken into two halves with similar assays with changes to methods described chronologically. The first experiment as described below was a two day fear conditioning procedure where data was obtained for both cued and contextual fear conditioning. Post fear testing animals were also placed into the open field where their locomotor activity was measured. Several KOR ligands were tested along with vehicle controls.

Subjects

Two hundred forty eight adult male C57BL/6J mice were obtained at approximately 8 weeks of age weighing 21-25 g (The Jackson Laboratory, Bar Harbor, ME) and were allowed to acclimate to the vivarium for approximately one week prior to commencement of testing. An N=8 was used for each experimental group (for each drug dose and its vehicle group). The mice were housed at a maximum of four per cage in an AAALAC-accredited animal facility with food (7012 Teklad LM-485 Mouse/Rat Sterilizable Diet, Harlan Laboratories, Inc., Indianapolis, IN) and water available ad libitum under a 12-h/12-h light/dark cycle (lights on at 0600 h to 1800 h) with all testing occurring during the light phase. All procedures were carried out in accordance with the “Guide for the Care and Use of Laboratory Animals” (Institute of Laboratory Animal Resources, National Academy Press, 1996) and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Drugs

The kappa opioid receptor (KOR) antagonists norbinaltorphimine (NorBNI) and (3R)-7-hydroxy-N-((1S)-1-[[3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl]-2-methylpropyl)-1,2,3,4-tetrahydro-3-isoquinolinecarboxamide (JDtic) (RTI International,
Research Triangle Park, NC) were administered 24H prior to training or testing as specified in results. While naloxone, morphine, buprenorphine, fentanyl HCL, U50,488, enadoline and diazepam (obtained from the National Institute on Drug Abuse, Rockville, MD) were administered 20min prior to testing. All drugs were dissolved in sterile 0.9% saline with the exception of diazepam (which was dissolved in 10% w/v 2-Hydroxypropyl-β-cyclodextrin (Sigma-Aldrich, St. Louis, MO) 90% sterile 0.9% saline). All drugs were injected subcutaneously in a volume equivalent to 10 ml/kg body weight.

**Choice of drugs and doses tested.** Benzodiazepines (used clinically as adjunctive treatments for PTSD) produce anxiolytic effects in the assays proposed here (Fraser et al., 2010; Sanger & Joly, 1985). For example, in the mouse, diazepam (0.54 mg/kg) reduced margin time in an open field assay (Fraser, et al., 2010). Also, in the mouse, diazepam (0.52 mg/kg) reduced duration of freezing in response to the conditioned stimulus in the PFC assay (Sanger & Joly, 1985; Smith, et al., 2010). Therefore, I propose using diazepam (0.1, 0.3, 0.56, and 1.0 mg/kg) as my control anxiolytic drug in these assays for experiment one.

The opioid drugs proposed below have a variety of binding affinities, with the benzodiazepine diazepam serving as the control comparison. The doses proposed are drawn from literature as cited and when possible from the same or similar behavioral assays in C57BL/6 mice. More specifically, morphine (0.1, 1.0, 3.0 and 10 mg/kg) and fentanyl (0.001, 0.01, and0.1 mg/kg) both have a high affinity for the MOR, where they act as agonists (Minami et al., 2009). MOR antagonist naloxone (0.1, 1.0, and 10 mg/kg) has a high affinity for MOR, though it has antagonist action at all subtypes of opioid receptor (Middaugh, Kelley, Cuisen, & Groseclose, 1999). Clinically, morphine and fentanyl are both used by the US armed services medical corps in the field for pain relief of injured personnel (Burnam, Meredith, Tanielian, & Jaycox, 2009)and so are especially relevant to ongoing influence on PTSD. The compounds U504880
(0.1, 1.0, and 10 mg/kg), enadoline (0.0001, 0.001, 0.01, and 0.1 mg/kg) (KOR agonists), and NorBNI (1.0, 10, 30 mg/kg) (KOR antagonist) have high binding affinity at the KOR which we have shown previously can effect stress related behaviors (Beardsley, et al., 2005; Bruchas, Land, Lemos, & Chavkin, 2009; Wang et al., 2009). The remaining drug buprenorphine (0.3, 1, and 3 mg/kg) has partial agonist activity at MORs and antagonist activity at KORs (Lelong-Boulouard et al., 2006). These drugs each represent either activation or inhibition of MORs and KORs as well as mixed MOR activation/KOR antagonism. This selection of drugs represents different actions that might underlay opioid influence on fear behavior.

**Apparatus**

Fear conditioning measurements for Experiment I were conducted using two commercially supplied, Near Infrared Video Freeze Systems controlling a total of seven individual test chambers (MED-VFC-NIR-M, Med Associates). Each test chamber consisted of a clear polycarbonate top and front, white acrylic back, and stainless steel sides, with a shockable grid floor (32 cm wide, 25 cm high, 25 cm deep; Med Associates Part Number VFC-008), enclosed in a white, sound-attenuated box (63.5 cm wide, 35.5 cm high, 76 cm deep; NIR-022MD), equipped with a speaker in the side wall. A proprietary light source (Med Associates NIR-100) provided near-infrared light (NIR; 940 nm). Video images of the behavioral sessions were recorded at a frame rate of 30 frames per second (640 × 480 pixels, downsampled within the driver to 320 × 240 pixels; about 1 pixel per visible mm²) via an IEEE 1394a (Firewire 400) progressive scan CCD video camera (VID-CAM-MONO-2A) with a visible light filter (VID-LENS-NIR-1) contained within each chamber and connected to a computer in the same room. Parameters for scoring were set to define freezing behavior as absence of movement for 1/15th second, and percent freezing was derived in real time from the video stream by computer software (Video Freeze; SOF-843) running on a Windows computer.
Locomotor measurements for Experiment I were conducted in eight commercially obtained, automated activity monitoring devices each enclosed in sound- and light-attenuating chambers that recorded distance travelled in cm in 10-m bins via computer-controlled circuitry (AccuScan Instruments, Columbus OH). The interior of each device was divided into separate 20x20x30 cm arenas permitting the independent and simultaneous measurement of two mice. Sixteen photobeam sensors per axis were spaced 2.5 cm apart along the walls of the chamber and were used to detect movement.

**Procedure**

The procedure used in Experiment I was synthesized following a review of broad methodologies published in the fear conditioning literature. A summary table of these methods is included in Table 1. This literature review revealed 32 unique sources or labs which had published at least two separate papers involving mouse fear conditioning. The parameters of most interest are habituation time, unconditioned stimuli time, aversive stimuli intensity and time, inter-trial interval used and number of trials.
Table 1

<table>
<thead>
<tr>
<th>Reference</th>
<th>Habituation (Event #1)</th>
<th>Unconditioned Stimulus (Event #2)</th>
<th>Aversive Stimulus (Event #3)</th>
<th>Shock Intensity</th>
<th>ITI</th>
<th>trial #</th>
</tr>
</thead>
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<tr>
<td>Abel, 2006</td>
<td>120 s</td>
<td>30 s</td>
<td>2 s</td>
<td>0.7 mA</td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td>Adams, 2002</td>
<td>140 s</td>
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<td>1 s</td>
<td>0.7 mA</td>
<td>60 s</td>
<td>3</td>
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<td>Anagnostaras, 2011</td>
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<td>30 s</td>
<td>2 s</td>
<td>0.77 mA</td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td>Anderson, 2010</td>
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<td>1 s</td>
<td>0.6 mA</td>
<td>20-180 s</td>
<td>4</td>
</tr>
<tr>
<td>Barad, 2005</td>
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<td>120 s</td>
<td>2 s</td>
<td>0.7 mA</td>
<td>120 s</td>
<td>2</td>
</tr>
<tr>
<td>Cain, 2004</td>
<td>120 s</td>
<td>120 s</td>
<td>2 s</td>
<td>0.7 mA</td>
<td>120 s</td>
<td>5</td>
</tr>
<tr>
<td>Caldarone, 2000</td>
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<td>30 s</td>
<td>2 s</td>
<td>0.5 mA</td>
<td>120 s</td>
<td>3</td>
</tr>
<tr>
<td>Comery, 2005</td>
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<td>15 s</td>
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<tr>
<td>Corbo, 2002</td>
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<td>20 s</td>
<td>3 s</td>
<td>0.75 mA</td>
<td>60 s</td>
<td>3</td>
</tr>
<tr>
<td>Davies, 2004</td>
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<td>33 s</td>
<td>3 s</td>
<td>0.75 mA</td>
<td>60 s</td>
<td>3</td>
</tr>
<tr>
<td>Davis, 2005</td>
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<td>2 s</td>
<td>0.57 mA</td>
<td>120 s</td>
<td>2</td>
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<td>Fanselow, 2010</td>
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<td>2 s</td>
<td>0.5 mA</td>
<td>180 s</td>
<td>3</td>
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<tr>
<td>Gulick, 2007</td>
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<td>30 s</td>
<td>2 s</td>
<td>0.57 mA</td>
<td>120 s</td>
<td>2</td>
</tr>
<tr>
<td>Held, 2007</td>
<td>300 s</td>
<td>30 s</td>
<td>2.5 s</td>
<td>0.4 mA</td>
<td>210 s</td>
<td>5</td>
</tr>
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<td>Holmes, 2008</td>
<td>180 s</td>
<td>30 s</td>
<td>2 s</td>
<td>0.6 mA</td>
<td>60-90 s</td>
<td>2</td>
</tr>
<tr>
<td>Imaki, 2009</td>
<td>300 s</td>
<td>20 s</td>
<td>1 s</td>
<td>1.0 mA</td>
<td>60 s</td>
<td>3</td>
</tr>
<tr>
<td>Kleppisch, 2008</td>
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<td>2 s</td>
<td>0.7 mA</td>
<td>N/A</td>
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<td>2 s</td>
<td>mA</td>
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<td>2 s</td>
<td>0.7 mA</td>
<td>90 s</td>
<td>4</td>
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<tr>
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<td>0.25 s</td>
<td>0.4 mA</td>
<td>120 s</td>
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</tr>
<tr>
<td>Maren, 2009</td>
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<td>1 s</td>
<td>1.0 mA</td>
<td>70 s</td>
<td>5</td>
</tr>
<tr>
<td>Minichiello, 2009</td>
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<td>30 s</td>
<td>2 s</td>
<td>0.5 mA</td>
<td>120 s</td>
<td>2</td>
</tr>
<tr>
<td>Nguyen, 2002</td>
<td>120 s</td>
<td>30 s</td>
<td>2 s</td>
<td>0.7 mA</td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td>Oitzl, 2011</td>
<td>180 s</td>
<td>20 s</td>
<td>2 s</td>
<td>0.4 mA</td>
<td>60 s</td>
<td>6</td>
</tr>
<tr>
<td>Palmiter, 2011</td>
<td>120 s</td>
<td>5 s</td>
<td>2 s</td>
<td>0.3 mA</td>
<td>40 s</td>
<td>100</td>
</tr>
<tr>
<td>Pape, 2003</td>
<td>120 s</td>
<td>10 s</td>
<td>1 s</td>
<td>0.2 mA</td>
<td>N/A</td>
<td>1</td>
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<tr>
<td>Seidenbecher, 2011</td>
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<td>9 s</td>
<td>1 s</td>
<td>0.45 mA</td>
<td>?</td>
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<td>Singewald, 2011</td>
<td>120 s</td>
<td>120 s</td>
<td>2 s</td>
<td>0.7 mA</td>
<td>120 s</td>
<td>5</td>
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<tr>
<td>Tonegawa, 2007</td>
<td>240 s</td>
<td>20 s</td>
<td>2 s</td>
<td>0.75 mA</td>
<td>120 s</td>
<td>2</td>
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<tr>
<td>Vargas-Irwin,</td>
<td>120 s</td>
<td>20 s</td>
<td>2 s</td>
<td>0.7 mA</td>
<td>60 s</td>
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<tr>
<td>unpublished data</td>
<td>180 s</td>
<td>20 s</td>
<td>2 s</td>
<td>0.75 mA</td>
<td>120 s</td>
<td>5</td>
</tr>
</tbody>
</table>

The habituation used by 80% of sources was 120 s long at the beginning of the first session. The most common unconditioned stimuli were white noise (65%) or tone (25%). Shock was almost universally (98%) used as the aversive stimuli. Inter-trial intervals (ITI) were much
more varied with 120 s being used 50% of the time while 60 s was being used 40% of the time. I
combined these most commonly used parameters for the use in the initial fear conditioning
procedure. This procedure consisted of Conditioning (Day 1) with Context and Cue tests (Day
2) that occurred across two, consecutive days. During Conditioning (Day 1), mice were placed in
the fear conditioning chambers and after a 2 m baseline period, three tone-shock pairings were
administered (60 s ITI), consisting of a 30 s white noise (80 dBA) co-terminating with a 2 s
scrambled footshock (0.70 mA, RMS, AC constant current) delivered through the grid floor,
followed by a 2 m rest period. Mice were then returned to their home cages. Each chamber floor
was then removed and replaced with a fresh unit and the chamber walls were cleaned with
unscented non-alcohol germicidal wipes (Sani-Cloth HB) prior to the next experimental session.
The next day during Context test (Day 2), mice were placed in the chambers and exposed to the
previous conditioning context for 5 m (without tone or shock deliveries) and then returned to
their home cage. Floors were changed and interiors were cleaned as above. New cage floors were
inserted along with white opaque plexi-glass pieces which made the floor smooth and the walls a
continuous curve. After 20 m had elapsed the mice were placed in this altered context for the
Cue test. Subjects were presented with a 2 m baseline period, three 30-s white noise (80 dBA)
noise presentations (60s ITI), followed by a 2 m rest period. Mice were then returned to their
home cage.

The locomotor procedure for part one of the experiments was conducted immediately
following the measurement of cue fear testing. Mice were removed from the fear conditioning
test chamber and moved across the room to the locomotor measurement chambers. Movement
was measured continuously and binned every 10 m for a total of 60 m then animals were placed
back into home cages. Variables recorded during this procedure were Total Distance traveled
(cm) and time spent (s) in the center and edges of the open field.
Data Analysis

Freezing was defined in parameters to be the absence of movement for 3 frames at a sample rate of 30 frames per second; 1/10\textsuperscript{th} of a second. Percent of time spent freezing was calculated relative to the rest of the session time and was used as the main dependent variable. It was recorded for the first 2 min of Conditioning as well as the first 5 min of Context Exposure and Test. More specifically, the first 2 min of initial chamber exposure freezing was calculated and used as a baseline measure. Freezing was calculated during the first five minutes of the Context Exposure session and is presented as a test of contextual freezing. Two-way repeated measures ANOVA followed by a Dunnett’s Test was used to compare percent freezing during the baseline measurement with those of the Context Exposure and Test sessions to determine if conditioning occurred, and was used to compare the experimental groups (dosage groups) with their vehicle controls to evaluate drug effects. An N=8 was used for each experimental group. This N was determined to have 90% power to detect a difference of means $\geq$ 12.00 in percent contextual freezing with a significance level (alpha) of 0.05 (one-tailed) calculated from results of a preliminary study comparing 10 mg/kg norBNI with vehicle-treated mice (N=8/group) (StatMate 2.0, GraphPad Software, Inc., 2004). All comparisons were considered statistically significant when P<0.05 and were conducted using commercial software (Prism 5.0c, GraphPad Software, Inc., 2004).

Initially, as an independent check of equipment accuracy, two observers hand scored the time spent freezing of a group of 8 mice on 3 consecutive days post fear conditioning. Freezing was defined to the observers as absence of movement except for respiration. Each observer observed the mice in real time and recorded time in seconds subjects spent freezing using a stopwatch. The time was totaled and divided by the total session time of 300 s to produce a percent freezing. The machine display was blocked so the observer was blind to the machine
score during their recording of the session. The machine scores for these same mice were compared to the two observers’ scores. A two-way ANOVA with repeated measures on both factors (observer and time) was conducted with simple effects between observers compared within each time period using a post-hoc, pair-wise Holm-Sidak test adjusted for multiple testing assuming one family for all tests (Prism 6 for Mac OSX, Version 6.0a.152, GraphPad Software Inc., San Diego, CA). There were no main effects for observer $F(2,14)=3.370, p>0.05$; or for time $F(2,14)=1.1797, p>0.05$. There was also no interaction between observer and time $F(4,28)=0.5436, p>0.05$. This indicates that the machine scoring of mouse freezing using the settings described above was consistent with observational measures of freezing.

For locomotor measurements during experiment one, distance travelled (cm) was subjected to analysis by a one-way ANOVA (4 levels of drug dose) followed by a Dunnett’s Post-Hoc Test comparing drug doses to the vehicle control group. Separately, time (s) spent in the center vs edge of the open field was analyzed via one-way ANOVA (4 levels of drug dose) with a Dunnett’s Post-Hoc Test comparing drug doses to the vehicle control group. All statistical tests were conducted using computer software (Prism 5d for Macintosh, GraphPad Software, Inc., San Diego, CA), and all types of comparisons were considered statistically significant if $p<0.05$.

**Results Experiment I**

**Preliminary studies for environment one.**

**Pilot Group.** The percent freezing for the pilot group (N=8) varied significantly from baseline in both contextual, $F(2, 21) = 5.907, p<0.001$, and cue, $F(2, 21) = 28.38, p<0.0001$, tests. Group differences indicated freezing was significantly higher than baseline in context ($p < 0.05$).
and cue ($p<0.001$) tests. While freezing was significantly lower during extinction for both context ($p < 0.05$) and cue ($p<0.001$) tests (Figure 1).

![Figure 1. Fear Conditioning Pilot group.](image)

This figure illustrates successful fear conditioning and extinction. Percent freezing during context and cue tests are significantly higher than baseline. Percent freezing after extinction training is significantly lower in both context and cue extinction conditions. Significance is denoted by * $p<0.05$, **$p<0.01$, and *** $p<0.001$.

**JDtic and Control Groups.** The percent freezing for the JDtic and control groups (N=8) varied significantly among the three different conditions, in both contextual, $F(2,21)= 6.222$, $p<0.01$, and cue, $F(2,21)=9.416$, $p<0.0001$, tests. Both initial and repeated time points are within subject and group differences indicated that the vehicle no shock control group froze significantly less than JDtic and vehicle only during the initial context test ($p<0.0001$) and not during the continued time course tests. While there was no significant difference between vehicle and JDtic contextual freezing at any time point (Figure 2).
Figure 2. Contextual Fear Conditioning JDtic and Control groups.

This figure illustrates that administration of the KOR antagonist JDtic increased contextual percent freezing more than vehicle or vehicle no shock at all time points except for the initial test day. The vehicle no shock group had significantly lower freezing than both JDtic and Vehicle treated groups on the initial test day. Significance is denoted by * $p$< 0.05, **$p$< 0.01, and *** $p$< 0.001.

Group differences for the cue freezing tests indicated that the vehicle no shock group froze significantly less ($p$< 0.001) than JDtic-treated groups at all time points measured except at baseline. The vehicle group froze significantly less ($p$< 0.05) than JDtic-treated groups on Day 7 while showing no difference to no shock at the same time point (Figure 3).
Figure 3. Cue Fear Conditioning JDtic and Control groups.
This figure illustrates that administration of the KOR antagonist JDtic increased cue percent freezing more than vehicle no shock at all time points. The JDtic treated group froze significantly more than either vehicle group on day 7. The vehicle no shock group had significantly lower freezing than both JDtic and Vehicle treated groups on all days but day 7. Significance is denoted by * \( p<0.05 \), ** \( p<0.01 \), and *** \( p<0.001 \).

NorBNI and Control Groups. The percent freezing for the NorBNI treated and control groups (N=8) varied significantly among the three different conditions, in both contextual, \( F(4,35)=17.918, p<0.0001 \), and cue, \( F(3,45)=9.416, p<0.0001 \), tests. Group differences indicated that the contextual freezing of the no shock vehicle group was significantly lower (\( p<0.001 \)) than the vehicle group only on the initial test day. While among the different NorBNI dosage groups (1, 10 and 30 mg/kg) on the initial test day, 1 and 10 mg/kg produced significantly higher (\( p<0.001 \)) freezing than either vehicle group or 30 mg/kg norBNI. The 10 mg/kg NorBNI group
had significantly higher freezing than all other groups on Days 7 ($p<0.001$) and 14 ($p<0.001$). There were no significant differences between groups’ freezing on Day 21 (Figure 4).

**Figure 4.** Context Fear Conditioning NorBNI and Control groups.

This figure illustrates that the administration of KOR antagonist norBNI significantly increased contextual freezing on the initial test day at 1 mg/kg and 10 mg/kg doses as compared to vehicle. The 10 mg/kg dose of norBNI increased contextual freezing during the 7 and 14 day time points as compared to vehicle. While the vehicle no shock treated group showed significantly less freezing than all other groups only on the initial test day. Significance is denoted by * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Group differences indicated that the cued freezing of the no shock vehicle group was significantly lower ($p<0.001$) than the vehicle group only during the Day 7 test. There were no significant differences amongst the different groups treated with NorBNI (1, 10 and 30 mg/kg) on the initial test day there were no significant differences. On Day 7 doses of 1 and 30 mg/kg produced significantly lower ($p<0.001$) freezing than vehicle treatment. On Day 14, the 10
mg/kg NorBNI treated group had significantly higher freezing than the other dosage groups of NorBNI but not the vehicle treated group ($p<0.001$). While on Day 21, NorBNI 30mg/kg displayed significantly higher freezing than vehicle treated groups (Figure 5).

![Graph showing freezing levels over time for different dosage groups of NorBNI and vehicle treatments.]

*Figure 5. Cue Fear Conditioning NorBNI and Control groups.*

This figure illustrates that administration of the KOR antagonist norBNI increased cued percent freezing more than vehicle or vehicle no shock only on day 14 (10mg/kg dose). The vehicle no shock group had significantly lower freezing than both norBNI and Vehicle treated groups on the day 7 only. While cue percent freezing was lower in the norBNI 30mg/kg treated group only on day 7. Significance is denoted by * $p<0.05$, **$p<0.01$, and *** $p<0.001$.

**Noise disturbances and stress assays.** The initial pilot work for the project took place in the fall semester of 2009 on the 6th floor of the R. Blackwell Smith Jr. Building. This information is significant because the kappa antagonist testing along with their control groups were tested starting in mid January of 2010, which is the month that VCU started an extensive renovation of the 1st, 2nd, and 5th floors of the R. Blackwell Smith Jr. Building. The vivaria for all subjects were located on the basement level of this same building. Construction in the
building too place both during working hours (0800-1700h) as well as after hours (1701-2000h). During the renovation on several separate occasions noise levels were measured in the lab space at as high as 85 dBA but ranging between 61–75 dBA during working hours (Scosche SPL 1000F 135DB Max SPL Meter) during the week of January 31-February 4, 2010.

The decibel levels measured in the lab space did not take into account sound frequencies that were below or above the human range of hearing that could also have been present during the use of the construction equipment. Previous research examining the detrimental effects of construction noise on breeding in mice and the difference in perception of construction noise show that mice are especially vulnerable to behavioral changes after exposure to such noise. Swiss Webster female mice that were exposed to 70, 80, or 90 dBA of cutting saw noise during the 1st, 2nd, or 3rd week of gestation had significantly higher numbers of stillborn pups regardless of the time of noise exposure (Rasmussen, Glickman, Norinsky, Quimby, & Tolwani, 2009). Further research that examined the perception of construction noise by different lab species including humans concluded that mice were significantly more likely to be adversely affected by and more likely to perceived construction noise that would go unnoticed by human workers (Norton, Kinard, & Reynolds, 2011). It is also likely that this noise could act as additional stressful stimuli that are beyond the control of the experimenter. This construction noise therefore introduced a confound that was likely to make data interpretation of the anxiety assays difficult if not impossible. The initiation of this construction and its possible influence on the anxiety assays was supported by the conflicting kappa pilot testing. This led me to the conclusion that either, tests would be put on hold until the completion of construction or equipment would need to be relocated to a new lab space.

Control studies from environment two.
The decision was made that since additional lab space was available and construction on the R. Blackwell Smith Jr. Building would continue for many months that the fear conditioning equipment would be moved to the nearby but unconnected Hunter Holmes McGuire Hall Annex. Once equipment was reinstalled and recalibrated new control groups were tested in environment two so that the methods could be revalidated in the absence of construction noise confounds.

The initial test groups completed in environment two were to test both the conditioning method’s effectiveness in the new environment and to serve as shock and no shock controls. Two groups were treated with vehicle and underwent conditioning only for one group the shock wasn’t administered. There were no significant differences in baseline freezing behavior. The groups were clearly different in both the context and cue tests on Day 2. Conditioning for the no shock group did not significantly increase conditioned freezing behavior. The shock group had significant increases in conditioned freezing. This is indicative of successful conditioning methods. This is supported by the data from original pilot group before noise confounds were introduced. Once these control groups were complete before additional testing took place there was much further thought about control procedures and additional assays available in the new environment.

In environment two lab space there were several open field locomotor apparatuses. The open field as previously mentioned is thought to model anxiety by utilizing rodents’ instinctive fear of open spaces and brightly lit environments (Archer, 1973; Rasmusson & Charney, 1997). For example, in the mouse, more time spent in open or well-lit space is thought to indicate less anxiety (Belzung & Griebel, 2001). Additionally the open field can be used as a measurement of the changes in locomotor activity related to the suppression or activation of locomotion. This second function of the open field is important as some of the drugs of interest in the fear conditioning assay have known effects on locomotor behavior. Since fear conditioning measures
conditioned immobility or freezing, drugs which effect the animals’ ability to locomote could confound the data measured in this assay. It was decided that this second assay would give a second layer of anxiety data as well as serve as a control for locomotor effects, and so would be administered after fear testing.

**Control data for environment two.**

**Fear control group.** New control groups were analyzed showing that vehicle shock and vehicle no shock control groups percent freezing differed significantly from each other $F(2,14) = 74.67, p<0.001$. Further, bonferroni post hoc tests indicated that the vehicle shock group showed significantly higher freezing in both contextual ($p<0.001$) and cue ($p<0.001$) tests while their baseline levels of freezing did not differ (Figure 6).

*Figure 6. Fear Conditioning Control groups.*

This figure shows the significantly higher level of contextual and cue percent freezing during testing for vehicle groups that were exposed to shock when compared to vehicle no shock treated groups. Significance is denoted by * $p<0.05$, ** $p<0.01$, and *** $p<0.001$. 

45
**Kappa Agonist Groups (acute).** The KOR agonist enadoline showed significant effects on freezing behavior, $F(3,26)= 75.04, p<0.001$. Enadoline significantly increased percent freezing during cue testing compared to vehicle at all doses tested ($p<0.001$). Further, enadoline increased freezing in contextual testing at both 0.01 and 0.1mg/kg ($p<0.001$) (Figure 7).

![Figure 7. Fear Conditioning Enadoline groups.](image)

This figure shows the significant dose dependant increase in freezing to both context and cue during FC testing after the administration of KOR agonist enadoline when compared to vehicle. Significance is denoted by * $p<0.05$, **$p<0.01$, and *** $p<0.001$.

Enadoline significantly affected locomotor behavior, $F(3,26)= 29.96, p<0.001$. Mice given 0.001 and 0.01mg/kg enadoline spent significantly more time in the center of the open field ($p<0.001$). The highest dose of enadoline 0.1 mg/kg significantly reduced total distance traveled in the open field ($p<0.001$) (Figure 8).
This figure shows the significant dose dependant decrease of total distance traveled after the administration of KOR agonist enadoline, as well as the significant dose dependant increase in time spent in the center of the open field when compared to vehicle treated groups. Significance is denoted by * \( p<0.05 \), ** \( p<0.01 \), and *** \( p<0.001 \).

The effects of an additional kappa agonist U50,488 were also analyzed in fear conditioning and open field. When U50,488 was administered there were significant effects on freezing behavior, \( F(3,28)=12.64, p<0.001 \). U50,488 significantly increased percent freezing during contextual and cue testing at 10 mg/kg, the highest dose tested compared to vehicle \( (p<0.001) \). Further, U50,488 also increased freezing during contextual testing at 0.1 mg/kg \( (p<0.001) \) (Figure 9).
Figure 9. Fear Conditioning U50,488 groups.

This figure shows the significant increase in freezing to both context (0.1 & 10 mg/kg) and cue (10 mg/kg) during FC testing after the administration of KOR agonist U50,488 when compared to vehicle. Significance is denoted by * $p<0.05$, **$p<0.01$, and ***$p<0.001$.

The KOR agonist U50,488, significantly affected locomotor behavior, $F(3,28)= 5.354$, $p<0.001$. All doses of U50,488 significantly decreased the time spent in the center of the open field ($p<0.001$) relative to vehicle control levels. The highest dose of U50,488 10 mg/kg significantly reduced total distance traveled in the open field ($p<0.001$) (Figure 10).
**Figure 10. U50,488 Open Field.**

This figure illustrates the significant decrease in total distance traveled (10 mg/kg) and the significant decrease in time spent in the center of the open field (all doses) for U50,488 treated groups when compared to vehicle treated groups. Significance is denoted by * $p<0.05$, **$p<0.01$, and ***$p<0.001$.

**Kappa Opioid Receptor Antagonist (acute).** The KOR antagonist NorBNI was examined and showed significant effects on freezing behavior, $F(3,28) = 2.021, p<0.001$. NorBNI significantly decreased percent freezing during contextual testing compared to vehicle at 10 mg/kg ($p<0.001$). The KOR antagonist did not significantly affect cued percent freezing (Figure 11).
Figure 11. Fear Conditioning NorBNI groups.

This figure shows the dose dependant decrease in freezing to context during FC testing after the administration of KOR antagonist norBNI (10 mg/kg) when compared to vehicle treated groups. Significance is denoted by * $p<0.05$, **$p<0.01$, and *** $p<0.001$.

The KOR antagonist affected locomotor behavior in the open field $F(3,28) 1.434$, $p<0.001$. All doses of NorBNI tested significantly reduced time spent in the center of the open field ($p<0.001$) but did not significantly affect total distance traveled (Figure 12).

Figure 12. NorBNI Open Field.
This figure illustrates the significant reduction in time spent in the center of the open field in NorBNI treated groups when compared to vehicle. Significance is denoted by * \(p<0.05\), **\(p<0.01\), and *** \(p<0.001\).

**Benzodiazepine/GABAA agonist.** The benzodiazepine diazepam was tested and had a significant effect on percent freezing, \(F(4,15)= 8.255, p<0.001\). Contextual percent freezing increased significantly after administration of 0.56 or 1.0 mg/kg diazepam (\(p<0.01\) and \(p<0.001\) respectively) compared to vehicle administration. However, cue freezing increased significantly only at 1.0 mg/kg diazepam (\(p<0.001\))(Figure 13).

![Figure 13. Fear Conditioning Diazepam Groups.](image)

This figure illustrates the significant increase in contextual (0.56 & 1 mg/kg) and cue (1 mg/kg) percent freezing during FC testing in diazepam treated groups when compared to vehicle treated groups. Significance is denoted by * \(p<0.05\), **\(p<0.01\), and *** \(p<0.001\).

Locomotor behavior in the open field was affected significantly by diazepam, \(F(4,15)=9.074, p<0.001\). The total distance traveled significantly decreased after administration of the 1.0
mg/kg diazepam dose ($p<0.001$). There were no significant effects of diazepam on center time compared to vehicle (Figure 14).

![Graph showing center time and total distance for different doses of diazepam](image)

**Figure 14. Diazepam Open Field.**

This figure illustrates the significant reduction in total distance traveled in the diazepam 1.0 mg/kg treated group when compared to vehicle treated groups. Time spent in the center of the open field for diazepam treated groups was not significantly different from vehicle treated groups. Significance is denoted by * $p<0.05$, **$p<0.01$, and *** $p<0.001$.

**Kappa Opioid Agonist Group (7 days after administration).** The same enadoline groups were tested for contextual and cue fear conditioning as well as open field locomotor 7 days after the original administration of drug and fear conditioning training. There were no significant differences between the enadoline treated groups and vehicle treat group in percent freezing $F(3,48)=3.129, p>0.05$. There were, however, still significant differences between enadoline treated groups and vehicle treated group in the open field. Enadoline treated groups spent significantly less time in the center of the open field, $F(3,48)=20.19, p<0.001$ (Figure 15)
at all doses tested 0.001 mg/kg ($p<0.001$), 0.01 mg/kg ($p<0.05$) and 0.1 mg/kg ($p<0.001$).

Although, there were no significant differences in total distance traveled between groups.

![Graph](image)

*Figure 15. Enadoline Open Field.*

This figure illustrates the significant reduction in time spent in the center of the open field is still present 7 days after the administration of enadoline when compared to vehicle treated groups. No significant changes in total distance traveled was present at the 7 day test point for enadoline treated groups when compared to vehicle treated groups.

Significance is denoted by * $p<0.05$, **$p<0.01$, and ***$p<0.001$.

The same U50,488 groups were tested for contextual and cue fear conditioning as well as open field locomotor 7 days after the original administration of drug and fear conditioning training. There were no significant differences between the enadoline treated groups and vehicle treatment group in percent freezing $F(3,56)=2.45$, $p>0.05$. There were, however, still significant differences between U50,488 treated groups and the vehicle treated group in the open field. U50,488 treated groups spent significantly less time in the center of the open field, $F(3,56)=7.247$, $p<0.0001$ (Figure 16) at all doses tested 0.1 mg/kg ($p<0.001$), 1mg/kg ($p<0.001$)
and 10 mg/kg ($p<0.05$). Although, there were no significant differences in total distance traveled between groups.

![Graph showing Center Time and Total Distance](image)

Figure 16. U50,488 Open Field.

This figure illustrates the significant reduction in time spent in the center of the open field for U50,488 treated groups, when compared to vehicle treated groups, is still present 7 days after administration. No significant lasting reductions to total distance traveled were observed at the 7 day test point. Significance is denoted by * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Discussion Experiment 1

Pilot and control data from environment one.

The significant differences between baseline context and extinction groups in the original pilot animals show that the initial methods result in effective conditioning of both contextual and cued fear in C57BL/6J mice. Further manipulation of methods was not attempted at this time and additional control groups for testing kappa receptor compounds were completed.

The next compound investigated was the kappa receptor antagonist JDTic along with two vehicle control groups; one control group with the aversive stimulus present (vehicle shock) and...
one with no aversive stimulus present (vehicle no shock). JDTic did not significantly reduce contextual freezing and only significantly reduced cued freezing at the 7 day test point. These results were disappointing given the anti-stress effects previously noted in other paradigms with this compound. However, more troubling than the lack of JDTic’s effects was the somewhat erratic results of the control groups which showed no differences to one another on several time points, even though one control group did not receive any exposure to the aversive shock stimuli.

Concurrent testing of a full dose effect curve of the different kappa receptor antagonist NorBNI with two similarly treated control groups resulted in the exacerbation of freezing behavior and also showed similar overlap between the shock and no shock vehicle groups. NorBNI significantly increased freezing behavior to both the conditioning context and cue at certain time points. The most interesting result was the dramatic increase in freezing behavior in the vehicle no shock control group to the conditioned cue on all but one time point tested.

**Acute kappa modulation of fear behavior.**

The kappa agonist enadoline significantly increased freezing in both cue and context conditions when administered subcutaneously prior to testing. This increase in freezing was dose dependant and occurred at doses where locomotor behavior was not significantly lower than vehicle. The highest dose tested significantly reduced the total distance traveled in the open field. While, a significant increase in the amount of time the animals spent in the center of the open field was observed at the intermediate and highest dose. The two different assays results support the anxiogenic profile of enadoline, and show that it specifically exacerbates conditioned freezing in C57BL/6J mice. Acute enadoline administration produces changes in locomotor behavior consistent with increased anxiety.

The kappa agonist U50,488 was also tested in both Pavlovian fear conditioning and the open field. The results with U50,488 were similar to those produced by enadoline though
slightly more erratic. There was significant exacerbation of freezing indicative of anxiogenic affect. Conversely, locomotor behavior indicated more time was spent in the center of the open field, which is normally interpreted to mean less anxiety. Again, both results occurred at doses that did not suppress locomotion. The highest dose tested of U50,488 suppressed locomotion and this marked suppression may explain the conflicting results with increased center time. The animals are normally placed in the center of the open field at the beginning of the test session. It is possible that their locomotion was so reduced that instead of moving to the edge of the open field as is normally observed, the mouse could not move from the center at all. This could also be interpreted as freezing in the fear conditioning test and so it is important to note this when trying to interpret data from this assay when locomotion is suppressed by the test compound.

The kappa antagonist norBNI was administered immediately after training due to its unique pharmacokinetics and testing took place as normal on day two. There was a significant decrease in freezing behavior at the intermediate dose tested but only in contextual freezing, cued freezing was not affected. Interestingly, total distance traveled was not affected but animals spent significantly less time in the center of the open field. This conflicting result between the two assays was the first sign that the current method of measurement, while serving as a control for gross locomotor effects, may not be an accurate measure of anxiogenic effects.

The kappa data may indicate that the open field when run as a within subjects assay after fear conditioning may be affected as much by the fear conditioning procedure as it is by the test compounds. There are also indications that at doses where locomotion is affected that freezing behavior and locomotor suppression may be indistinguishable in the Pavlovian fear conditioning assay. This shows that controlling for locomotor confounds is important in interpreting this data. This confound concern becomes more apparent when testing a benzodiazepine.

**Acute benzodiazepine modulation of fear behavior.**
The benzodiazepine and GABA$_A$ agonist diazepam was tested and significantly exacerbated freezing behavior in both contextual and cued conditions. However context fear was affected at the intermediate dose while cue was affected only by the highest dose tested. Additionally, locomotor behavior shifted away from the center of the open field with significant reductions in total distance traveled at the highest dose tested. The data from both assays seem to indicate that acutely diazepam, at the doses tested, contrary to expectations produces anxiogenic effects.

Diazepam has long been used as an anxiolytic drug in humans (trade name Valium®) and shows similar effects in other mammals including mice (Boissier, Simon, & Aron, 1968). Further exploration of the literature show that the anxiolytic effects of diazepam and other benzodiazepines are sensitive to prior experience in the testing context in other anxiety assays like elevated plus maze and light dark box (Holmes, Iles, Mayell, & Rodgers, 2001; Rodgers & Shepherd, 1993). Prior experience in the anxiety inducing test environment lessens the anxiolytic effects of diazepam (Holmes, et al., 2001; Rodgers & Shepherd, 1993). This may be the effect we are observing here as the Pavlovian fear conditioning methods depend on the previous conditioning of the test environment paired with an aversive stimulus prior exposure is necessary before testing.

The conflicting and unexpected data resulting from these two assays in conjunction was of concern. Locomotor effects were important confounds to control for and yet the second assay using the open field was not yielding results which clarified the data. Instead the open field used as a within subject assay seemed to further complicate the data. Groups were tested under the acute influence of drug effects which included the possible confound of locomotor suppression. In an attempt to clarify the effects of the acute drug exposure on fear behavior without the confounding locomotor suppression testing was conducted again in the kappa agonist groups.
when the animals were in a non-drug state one week post conditioning, in the groups that had already undergone testing.

**Lasting changes to baseline locomotor behavior.**

There were no lasting significant effects to conditioned freezing behavior. The open field still showed a significant reduction of the time spent in the center area. This implies a lasting increase to basal levels of anxiety. The vehicle groups did not display the lasting changes and instead showed an almost equal distribution of time spent in the center versus the edge of the open field. However, it is impossible to conclude that this change to level of anxiety like behavior is due completely to the administration of the kappa agonists alone. It may have been a combination of the anxiogenic activation of the kappa system in conjunction with the conditioning that led to this change. It also may have been the combination of the kappa activation and the pre-exposure to the locomotor chambers. These data suggest that an interaction occurred in one of three ways. Either, the drug effected locomotor behavior directly obscuring freezing data interpretation. The fear conditioning training effected locomotor behavior. Or the pre-exposure to the locomotor or conditioning context had an effect on final testing.

The overall change in baseline behavior well after drug exposure is fascinating. The investigation of fear conditioned animals in a non-drug state after drug exposure is important in the context of PTSD in that the phenomenon is long lasting and subject to spontaneous reoccurrence. In further study regarding modifying methods to remove locomotor confounds as well as incorporate a non-drug state a publication by Cain, et al. (2004) seemed to offer better methods choices. There was a test day comparable to my original methods, extinction exposure training, and an additional non-drug test period (Cain, Blouin, & Barad, 2004).

**Transition to new methods.**
I decided to adopt procedures that were a synthesis of both the Cain procedure and my original methods. This synthesis of procedures allowed for a direct comparison of my previous data (Day 2) as well as a data point free of drug locomotor influence (Day 3). An additional gain by using these procedures was the exposure learning extinction component. While the day three extinction measures gave a non-drug state data point, it was also after an extended exposure to the training environment. This adds to the preclinical model a data point that could be used as a comparison to drug administration during exposure therapy in clinical settings. While this model uses rodents, it has been observed that the same memory processes (Siegmund & Wotjak, 2006) that occur during extinction learning are mirrored in humans and that the extinction of conditioned fear has served as the explicit model for behavior therapy of human anxiety disorders (Craske, 1999; Davis, 2011; Wolpe, 1969). The process of the progressive weakening of the conditioned response by repeated presentations of CS without the US, is the basis of exposure therapy (Cain, et al., 2004). This added component can be used to postulate not only the mechanism of opioids influence on fear but how use of these analgesics could impact treatment of PTSD.

Within the new procedures approach I decided to concentrate on the contextual fear component (though there is one measurement of cue fear effects). The brain areas involved in contextual fear conditioning are primarily the hippocampus and the amygdala (Anagnostaras, Gale, & Fanselow, 2001). Infusion of the NMDA receptor antagonist D,L-2-amino-5-phosphonovaleric acid (APV) into the hippocampus is sufficient to block the acquisition of contextual fear (Young, Bohenek, & Fanselow, 1994). The local infusion of APV into the basolateral amygdala blocks the acquisition of tone or contextual fear (Campeau, Miserendino, & Davis, 1992; Fanselow & Kim, 1994; Maren, Aharonov, Stote, & Fanselow, 1996). Lesion studies support the necessary involvement of both these brain areas and the connections between
them, high-frequency stimulation of the pathways carrying information from the hippocampus to the amygdala produces LTP, and lesions of these projections selectively block the acquisition of contextual fear (Maren, 1996; Maren & Fanselow, 1995).

Opioids have been linked to inhibition of major neurotransmitters in the hippocampus and the amygdala (Wagner, 1996). Mu agonists have been shown to inhibit the release of norepinephrine and acetylcholine in the hippocampus (Jackisch, Geppert, Brenner, & Illes, 1986; Jackisch, Geppert, & Illes, 1986; Werling, Brown, & Cox, 1987). Kappa agonists have also been shown to inhibit the release of both norepinephrine and acetylcholine (Jackisch, Geppert, Brenner, et al., 1986; Jackisch, Geppert, & Illes, 1986; Werling, et al., 1987). Using a mossy fiber synaptosomal preparation, very high concentrations of kappa agonist could inhibit both dynorphin and glutamate release (Gannon & Terrian, 1991). The decision to focus on contextual fear after the change in methods I believe is well supported by the known distribution of opioid receptors in both the hippocampus and amygdala as well as the interconnected functions of these two brain areas. It is further supported by the evidence of the suppression by opioids of key neurotransmitters like glutamate and norepinephrine that are involved in the learning process (Gannon & Terrian, 1991; Jackisch, Geppert, Brenner, et al., 1986; Jackisch, Geppert, & Illes, 1986; Werling, et al., 1987).

Another change in the new methods is the use of a measurement of level of movement while the animals are in the conditioning chambers, the motion index. As explained in the data analysis portion of methods the motion index is the number of pixels that have changed within a designated time period more than they would change if the mouse was not present (i.e., video noise). A calibration is run at the beginning of each session prior to the subject being placed in the chamber and that video is used with that session as the comparison video. In other words each session has its own individual reference video used as the baseline control. Since this
motion index is being generated in chamber during the fear sessions this removes some confounds associated with measuring locomotor behavior post session in a different environment. One reason locomotor testing was introduced was the concern for drug induced locomotor effects. However, I found that measurement of those effects in a new environment some time after drug administration and conditioning made for interesting if difficult to interpret data. The data indicate that there was an impact on locomotor activity; however, it is difficult to know if this difference was due to the conditioning or the drug administration. Another concern was the difference in drug time course. Initially locomotor activity was measured after both test session had already occurred so a minimum of 51 minutes had elapsed since drug administration. While this may not be a problem for some of the longer acting drugs it is certainly possible that the drug would no longer be acting centrally by the time locomotor was measured. The motion index when used as a measurement of general movement can be indicate overall levels of activity during the conditioning and test sessions which removes the concern of drug time course and measurement in a new environment.
Change in Methodology.

Refining locomotor activity measurements. During the measurement and analysis of part one of experiments several challenges which complicated and possibly confounded data interpretation were recognized. The first of these challenges was the interpretation of locomotor data. Animals were first tested in the Near Infrared Video Freeze Systems, and then placed in the Open Field for locomotor measurements. This initiated two concerns. One was the change of context in which the animals’ behavior was being measured. Previous reports indicate that contextual cues specifically influence the severity of freezing behavior and that measurement in a novel context can be used to measure the degree of generalizability of the conditioned fear response (Gonzalez, Quinn, & Fanselow, 2003). In changing the context that locomotor behavior is measured in, the data are less clearly interpreted with regard to the fear conditioning tests. The second challenge is the possibility that the fear conditioning has, in itself, modified basal levels of open field locomotor activity. Though there are no direct data in mice showing a modification of open field activity by fear conditioning, it has been shown that activity in these two assays share genetic framework (Sokoloff, Parker, Lim, & Palmer, 2011). The proposed change in methods would use a movement measure that is recorded during the fear conditioning session, which would remove this concern. Additionally, the time course in which the components of the experiments take place (20 m, 40 m and 47 m post injection) give rise to the concern that there are different amounts of central drug activity taking place during the fear tests than is taking place during the locomotor tests. With these challenges in mind I shifted to using the Motion Index analysis described below.
Refining contextual fear measurements. A second concern was that in working with both cue and contextual fear tests it became clear that the drugs administered were having differential effects in the two tests. While both sets of results were interesting and continued exploration of the cue fear response is warranted, I chose to focus on contextual fear conditioning in a new modified procedure. These methods were based on the methods in a report that focused not only on acute contextual fear but on drug effects during both acute contextual freezing and a measure of extinction (Cain, et al., 2004). This additional testing condition occurs in an absence of acute drug activity. The new methods outlined below provide a measurement of contextual freezing and the influence of drugs administered before this test that is comparable to the previous methods. Additionally the animals are exposed to the conditioning context that is then measured as a level of extinction learning and how it was affected by the drug administration. This testing occurs in a non-drug state and provides data on the effects of acute drug administration while avoiding the complication of acute drugs effects on movement. Also, as described above, contextual fear conditioning involves the hippocampus as well as the amygdala. One of the ways I postulate opioids may be influencing fear behavior by their effects on learning itself, this would most likely involve a hippocampus-dependant mechanism. Based both on the preliminary data with the experiments in part one, as well as a desire to more closely replicate the methods presented in (Cain et al., 2004) I chose to focus on contextual freezing of Pavlovian fear conditioning.

I hypothesize that opioid compounds with MOR agonist properties will decrease fear behavior in Pavlovian fear conditioning and facilitate extinction.

Also, because of previous research involving KOR antagonists;

I hypothesize that compounds with KOR antagonist properties will decrease fear behavior in Pavlovian fear conditioning and facilitate extinction.
Methods Experiment II

The following is the second of two experiment methods. The second experiment as described below is a three day fear conditioning procedure where data were obtained for contextual fear conditioning and its extinction. Animal’s activity was measured in real time in the fear conditioning chambers. Several KOR ligands and MOR ligands were tested along with vehicle controls.

Subjects

Four Hundred sixty four adult male C57BL/6J mice were used in Experiment II with all other aspects of their treatment remaining the same as in Experiment I.

Drugs

The KOR antagonist norbinaltorphimine (NorBNI) (RTI International, Research Triangle Park, NC) was administered 24H prior to training or testing as specified in results. While naloxone, morphine, buprenorphine, fentanyl HCL, U50,488, enadoline and diazepam (obtained from the National Institute on Drug Abuse, Rockville, MD) were administered 20min prior to testing. All drugs were dissolved in sterile 0.9% saline with the exception of diazepam (which was dissolved in 10% w/v 2-Hydroxypropyl-β-cyclodextrin (Sigma-Aldrich, St. Louis, MO) in 90% sterile 0.9% saline). All drugs were injected subcutaneously in a volume equivalent to 10 ml/kg body weight.

Choice of drugs and doses tested. Drugs used in experiment two are justified in the methods for experiment one. All is the same with the exception of the removal of JDtic and diazepam from testing.

Apparatus
Fear conditioning measurements for Experiment II as well as locomotor measurements for Experiment II were conducted using two commercially supplied, Near Infrared Video Freeze Systems as described in Experiment I.

**Procedure**

The procedure used in Experiment II was a modification of those described in (Cain, et al., 2004) and consisted of a Conditioning (Day 1), Context Exposure (Day 2) and a Test (Day 3) that occurred across three, consecutive days. During Conditioning (Day 1), mice were placed in the fear conditioning chambers and after a 2-min baseline period, three tone-shock pairings were administered (60s ITI), consisting of a 30-s white noise (80 dBA) co-terminating with a 2-s scrambled footshock (0.70 mA, RMS, AC constant current) delivered through the grid floor, followed by a 2-min rest period. Mice were then returned to their home cages. Each chamber floor was then removed and replaced with a fresh unit and the chamber walls were cleaned with unscented non-alcohol germicidal wipes (Sani-Cloth HB) prior to the next experimental session. The next day during Context Exposure (Day 2), mice were placed in the chambers and exposed to the previous conditioning context for 20 min (without tone or shock deliveries) and then returned to their home cage. During the Test on Day 3, mice were placed in the chambers for 5 min as a measurement of the extinction of contextual freezing. Percent freezing was recorded as the dependant variable and was analyzed as is described in the next section.

Locomotor measurements during part two of testing were simultaneously recorded along with freezing and are presented as the Motion Index (MI). The MI represents a general activity level of the animal and is used as a measure to control for direct motor effects of the drugs being tested. As a control for drug effects only the MI on Day 2 was analyzed (this period directly followed drug administration). This Index was recorded as a dependant variable and was computed and analyzed as described in the next section.
To ascertain the persistence of drug effects on extinction, time course testing was conducted. This testing used the same methodology as described above with a Conditioning (Day 1), Context Exposure (Day 2) but with varied durations before the Test day. For the drug enadoline the dose (0.1 mg/kg) which significantly facilitated extinction was tested using separate groups of mice for each time point test day. The different time points were Day 3, Day 7, and Day 14. Drug administration occurred just as with previous methods preceding Context Exposure on conditioning Day 2.

A time course of effects was also generated for the norBNI dose (30 mg/kg) which significantly facilitated extinction. Due to the unique pharmacokinetics of norBNI (Endoh, Matsuura, Tanaka, & Nagase, 1992) drug administration occurred immediately post conditioning on day 1 and the time course was extended to include Day 21 and Day 28 test groups.

**Data Analysis**

Freezing was defined to be the absence of movement for 3 consecutive frames at a sample rate of 30 frames per second; 0.10 of a second. Percent of time spent freezing was calculated relative to the rest of the session time and was used as the main dependent variable. It was recorded for the first 2 m of Conditioning as well as the first 5 m of Context Exposure and 5 m on Day 3 Test. More specifically, the first 2 m of initial chamber exposure freezing was calculated and used as a baseline measure. Freezing was calculated during the first 5 m of the Context Exposure session and are presented as a test of contextual freezing and the five minutes of test on Day 3 are recorded and presented as a test of extinction of contextual freezing. Two-way repeated measures ANOVA followed by a Dunnett’s Test were used to compare percent freezing during the baseline measurement with those of the Context Exposure and Test sessions to determine if conditioning occurred, and was also used to compare the experimental groups (dosage groups) with their vehicle controls to evaluate drug effects. An N=8 was used for each experimental
group. This N was determined to have 90% power to detect a difference of means ≥ 12.00 in percent contextual freezing with a significance level (alpha) of 0.05 (one-tailed) calculated from results of a preliminary study comparing 10 mg/kg norBNI with vehicle-treated mice (N=8/group) (StateMat 2.0, GraphPad Software, Inc., 2004). All comparisons were considered statistically significant when p<0.05 and were conducted using commercial software (Prism 5.0c, GraphPad Software, Inc., 2004).

For locomotor measurement during part two of experiments, were conducted via a proprietary motion analysis algorithm that was used to generate a Motion Index from the digital video stream in order to estimate the amount of mouse movement. This algorithm analyzed the video stream in real time, as it was being saved to disk, and it was capable of analyzing up to four video cameras simultaneously recording at 30 frames per second, 320 × 240 pixels, 8-bit grayscale. Briefly, a reference video sample is taken prior to placing the mouse into the chamber (“calibration”). This reference sample establishes the amount of baseline noise inherent in the video signal on a per pixel basis, across multiple successive frames. Once the mouse is placed in the chamber, successive video frames are continuously compared to each other and to the reference sample on a pixel by pixel basis. Any differences between pixels in the current video signal larger than those in the reference sample are interpreted as animal movement. These differences (in pixels) are summed for each image frame, and this summation is counted as the Motion Index. The Motion Index is the number of pixels that have changed within a designated time period more than they would change if the mouse was not present (i.e., video noise). For video storage, the four streams from the four chambers are saved into one Windows Media Video 9 file (WMV3 codec), 320 × 240 pixels (32 bits) per stream, 30 frames, with a variable total bitrate averaging about 1200 kb/s. Motion Index numbers were analyzed in a one-way between subjects ANOVA (4 levels of drug dose) with a Dunnett’s Test post hoc analysis
comparing drug doses to the vehicle control group. All statistical tests were conducted using computer software (Prism 5.0c, GraphPad Software, Inc., San Diego, CA), and all types of comparisons were considered statistically significant if p<0.05.

Results Experiment II

Experiment II drug studies.

KOR Agonists on Contextual Fear. The KOR agonist enadoline was tested and significantly increased contextual freezing $F(4,35)= 6.736, p< 0.001$ on Day 2 (of the new methodology). Post hoc analysis revealed that the two highest doses of 0.01 mg/kg and 0.1 mg/kg significantly increased freezing behavior compared to vehicle on Day 2 (Figure 17). The highest dose of enadoline (0.1 mg/kg, $p<0.01$) also significantly facilitated the effects of exposure extinction training on Day 3 $F(4,35)= 6.736, p< 0.001$ (Figure 17).

![Figure 17. Enadoline Contextual Fear.](image)

This figure illustrates that the percent freezing to context was significantly increased on Day 2 after administration of the KOR agonist enadoline (0.01 & 0.1 mg/kg) when compared to vehicle. Also, when enadoline is administered prior to extinction training the 0.1 mg/kg dose significantly facilitated the extinction of contextual conditioned
freezing that was measured on Day 3 verses vehicle treated groups. Significance is denoted by * $p<0.05$, **$p<0.01$, and ***$p<0.001$.

There were also significant differences between enadoline treated groups and vehicle treated groups in motion index scores on Day 2 $F(4,19)=15.06, p<0.0001$. The highest two doses of enadoline (0.01 mg/kg and 0.1 mg/kg, $p<0.0001$) significantly reduced motion index scores compared to vehicle on Day 2 (Figure 18).

![figure 18](image)

**Figure 18.** Enadoline Activity Data.

This figure illustrates the significant dose dependant reduction in the activity scores that were measured on Day 2 conditioning in enadoline verses vehicle treated groups.

Significance is denoted by * $p<0.05$, **$p<0.01$, and ***$p<0.001$.

The KOR agonist U50,488 was tested and did not significantly increase contextual freezing $F(3,28)=1.254, p<0.3092$ on Day 2 (Figure 19). All doses of U50,488 (0.1 mg/kg, $p<0.05$, 1.0 mg/kg $p<0.01$, and 10 mg/kg $p<0.001$) significantly facilitated the effects of exposure extinction training on Day 3 $F(3,28)=65.37, p<0.0001$ (Figure 19).
Figure 19. U50,488 Contextual Fear.

This figure shows that there was no significant difference in freezing to the conditioned context in KOR agonist U50,488 treated groups when compared to vehicle treated groups. However, in groups that received U50,488 on Day 2 during extinction training there was a significant facilitation of the extinction of freezing to the context when compared to vehicle treated groups. Significance is denoted by * \( p<0.05 \), ** \( p<0.01 \), and *** \( p<0.001 \).

There were also significant differences between U50,488 treated groups and vehicle treated groups in motion index scores on Day 2 \( F(3,28)=4.500, p<0.05 \). The highest (10 mg/kg, \( p<0.01 \)) and lowest doses (0.1 mg/kg, \( p<0.05 \)) of U50,488 significantly reduced motion index scores compared to vehicle on Day 2 (Figure 20).
**Figure 20. U50,488 Activity Data.**

This figure illustrates the significant reduction in the activity scores that were measured on Day 2 conditioning in U50,488 treated groups (0.1 mg/kg & 10 mg/kg) verses vehicle treated groups. Significance is denoted by * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

**KOR Antagonists on Contextual Fear.** The KOR antagonist nor-BNI was tested and significantly reduced contextual freezing $F(3,28)=7.054$, $p<0.01$ on Day 2. Dunnett’s post hoc analysis revealed that the all three tested doses 1 mg/kg, 10 mg/kg and 30mg/kg significantly (p<0.001) decreased freezing behavior compared to vehicle on Day 2 (Figure 21). The highest dose of norBNI (30 mg/kg, $p<0.01$) also significantly facilitated the effects of exposure extinction training on Day 3 (Figure 21).
Figure 21. NorBNI Contextual Fear.

This figure illustrates the significant reduction in percent contextual conditioned freezing on Day 2 in all norBNI treated groups when compared to vehicle. Also, shown is the facilitation of extinction by the either; the administration of norBNI 30mg/kg before extinction training, or its continued activity maintaining a significantly lower freezing percentage than vehicle treated groups on Day 3. The interpretation of Day 3 data is complicated by norBNI’s extended time course of effects in vivo. Significance is denoted by * $p<0.05$, **$p<0.01$, and ***$p<0.001$.

There were also significant differences between norBNI treated groups and vehicle treated groups in motion index scores on Day 2 $F(3,28)= 2.664, p<0.05$ (Figure22). The highest dose 30 mg/kg, ($p< 0.05$) significantly raised motion index scores compared to vehicle on Day 2.
Figure 22. NorBNI Activity Data.

This figure illustrates the significant increase in the activity scores that were measured on Day 2 conditioning in norBNI 30 mg/kg treated verses vehicle treated groups. Significance is denoted by * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

**Time Course of Contextual Fear Extinction.** The KOR agonist enadoline was tested to see if the facilitation of extinction persisted over time, compared to vehicle. The 0.1 mg/kg dose of enadoline significantly facilitated extinction training $F(3,36)=168.8, p<0.0001$, evident by significantly less freezing on Days 3 and 7 ($p<0.001$) as well as Day 14 ($p<0.01$) when compared to vehicle (Figure 23).
Figure 23. Enadoline Extinction Time Course.

This figure shows the contextual freezing percentages of six separate groups that experienced Day 1 conditioning and Day 2 exposure with an additional test on either Day 3, Day 7 or Day 14. Three groups received enadoline 0.1 mg/kg on Day 2 and then varied time point tests; while three other groups received vehicle on Day 2 and then varied time point tests. Regardless of the time point at which extinction of freezing was measured the enadoline 0.1 mg/kg treated groups showed significantly lower freezing than vehicle treated groups. Significance is denoted by * $p<0.05$, **$p<0.01$, and *** $p<0.001$.

The KOR antagonist norBNI was tested to see if the facilitation of extinction was stable over time, compared to vehicle. The 30 mg/kg dose of norBNI significantly facilitated extinction training $F(5,70)= 58.20$, $p<0.0001$, evident by significantly less freezing on Days 3, 7, 14, 21, and 28 ($p<0.001$) when compared to vehicle (Figure24).
This figure shows the contextual freezing percentages of ten separate groups that experienced Day 1 conditioning and Day 2 exposure with an additional test on either Day 3, 7, 14, 21 or 28. Five groups received norBNI 30 mg/kg immediately after conditioning on Day 1 were tested on Day 2 and then received varied time point tests; while five other groups received vehicle immediately after conditioning on Day 1 were tested on Day 2 and then had varied time point tests. Regardless of the time point at which extinction of freezing was measured the norBNI 30 mg/kg treated groups showed significantly lower freezing than vehicle treated groups. Significance is denoted by * \( p<0.05 \), ** \( p<0.01 \), and *** \( p<0.001 \).

**KOR Antagonist Blocking of KOR Agonist Effects.** The KOR antagonist norBNI was tested in conjunction with enadoline to determine if the behavioral effects were KOR mediated. NorBNI pre-treatment blocked, \( F(4,35)=0.6191, p>0.05 \), enadoline’s significant acute exacerbation of conditioned freezing on Day 2 (Figure 25). Furthermore, co-administration of norBNI blocked, \( F(4,35)=0.6191, p>0.05 \), enadoline’s significant facilitation of extinction on Day 3 (Figure 26).
Figure 25. NorBNI+ Enadoline Contextual Fear.

This figure illustrates the percent conditioned freezing that was measured on Day 2 in both groups which received enadoline plus vehicle, as well as, groups that received 10 mg/kg norBNI immediately after conditioning on Day 1 and then one of four doses of enadoline on Day 2. The pretreatment of 10 mg/kg norBNI blocked the significant exacerbation of contextual freezing by the administration of enadoline that was observed in enadoline/vehicle treated groups. Significance is denoted by * \( p<0.05 \), ** \( p<0.01 \), and *** \( p<0.001 \).
Figure 26. NorBNI+ Enadoline Contextual Fear.

This figure illustrates the percent conditioned freezing that was measured on Day 3 in both groups which received enadoline plus vehicle, as well as, groups that received 10 mg/kg norBNI immediately after conditioning on Day 1 and then one of four doses of enadoline on Day 2. The pretreatment of 10 mg/kg norBNI blocked the significant facilitation of the extinction of contextual freezing by the administration of 0.1 mg/kg enadoline that was observed in enadoline+vehicle treated groups. Significance is denoted by * $p<0.05$, **$p<0.01$, and *** $p<0.001$.

The pretreatment of 10 mg/kg norBNI also blocked, $F(5,42)=1.860$, $p>0.05$, enadoline’s significant motion index reduction on Day 2 as well as having no significant effects of its own on the motion index of Day 3 (Figure 27).
Figure 27. NorBNI + Enadoline Activity Data.

This figure illustrates the activity levels that were measured on Day 2 and Day 3 conditioning in norBNI+enadoline treated groups versus enadoline+vehicle treated groups. No significant differences were observed in activity levels. Significance would be denoted by * \( p<0.05 \), ** \( p<0.01 \), and *** \( p<0.001 \).

**MOR Agonist Effects on Contextual Fear.** The Mu agonist fentanyl was tested and the intermediate dose of 0.01 mg/kg significantly reduced freezing, \( F(3,28)=11.67, p<0.001 \) on Day 2 (Figure 28). While on Day 3 all three doses of fentanyl (0.001 and 0.01 mg/kg, \( p<0.001 \); 0.1 mg/kg, \( p<0.01 \)) significantly facilitated extinction learning (Figure 28). The intermediate dose of fentanyl, 0.01 mg/kg, significantly increased, \( F(3,28)=38.51, p<0.0001 \), the motion index score on Day 2 of conditioning compared to vehicle (Figure 29).
Figure 28. Fentanyl contextual fear.

This figure illustrates the significant reduction in percent contextual conditioned freezing on Day 2 in the fentanyl 0.01 mg/kg treated group when compared to vehicle. Also shown is the significant facilitation of the extinction of contextual conditioned freezing in all fentanyl treated groups when compared to vehicle treated groups on Day 3.

Significance is denoted by * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Figure 29. Fentanyl Activity Data.
This figure illustrates the significant increase in activity levels that was measured on Day 2 of conditioning in the fentanyl 0.01 mg/kg treated group versus vehicle treated groups. Significance is denoted by * $p<0.05$, **$p<0.01$, and *** $p<0.001$.

The MOR agonist morphine was tested and significantly reduced freezing on Day 2, $F(4,35)= 13.66$, $p<0.0001$, at the highest two doses of 3 mg/kg and 10 mg/kg (Figure 30). Morphine also significantly facilitated extinction shown on Day 3 at three doses (1 mg/kg and 3 mg/kg $p<0.01$; and 10 mg/kg, $p<0.001$) (Figure 30). Morphine also significantly increased motion index scores over vehicle on Day 2 but only at the highest dose tested, 10 mg/kg, $p<0.001$ (Figure 31).

**Figure 30. Morphine Contextual Fear.**

This figure illustrates the significant reduction in percent contextual conditioned freezing on Day 2 in morphine treated groups (3 & 10 mg/kg) when compared to vehicle. Also shown is the significant facilitation of the extinction of contextual conditioned freezing by the administration of morphine (1, 3 & 10 mg/kg) before extinction training when compared to vehicle treated groups on Day 3. Significance is denoted by * $p<0.05$, **$p<0.01$, and *** $p<0.001$. 

80
Figure 31. Morphine Activity Data.

This figure shows the significant increase in activity levels that was measured on Day 2 of conditioning in the 10 mg/kg morphine treated group verses vehicle treated groups. Significance is denoted by * $p<0.05$, **$p<0.01$, and ***$p<0.001$.

**MOR antagonist effects on contextual fear.** MOR antagonist naloxone was tested and did not significantly affect freezing behavior, $F(3,28)=0.4814, p=0.6979$, compared to vehicle on either Day 2 or Day 3 (Figure 32). Naloxone also had no significant affects on motion index scores, $F(3,28)=1.441, p=0.5207$, on Day 2 compared to vehicle (Figure 33).
Figure 32. Naloxone Contextual Fear.

This figure illustrates the no change in percent contextual conditioned freezing on Day 2 in naloxone treated groups when compared to vehicle treated groups. Also, shown is the lack of a significant effect on the extinction of contextual conditioned fear in either naloxone or vehicle treated groups. Significance would be denoted by * $p<0.05$, **$p<0.01$, and *** $p<0.001$.

Figure 33. Naloxone Activity Data.
This figure illustrates that there was no significant difference in activity level that was measured on Day 2 of conditioning in naloxone treated groups verses vehicle treated groups. Significance would be denoted by * $p<0.05$, **$p<0.01$, and *** $p<0.001$.

**MOR Antagonist Blocking of MOR Agonist Effects.** MOR antagonist naloxone (1.0 mg/kg) was co-administered with morphine to determine if the agonist’s effects could be blocked. The co-administration of naloxone with morphine blocked, $F(4,35)= 3.96, p=0.5714$, morphine’s significant acute reduction of conditioned freezing on Day 2 (Figure 34). Furthermore, co-administration of naloxone blocked, $F(4,35)= 3.96, p=0.5714$, morphine’s significant facilitation of extinction on Day 3 (Figure 35). Morphine’s significant increase in motion index level on Day 2 was also blocked $F(4,35)= 0.2773, p=0.2114$, by the co-administration of naloxone (Figure 36).

![Figure 34. Naloxone + Morphine Acute Contextual Fear.](image)

This figure illustrates the percent conditioned freezing that was measured on Day 2 in groups which received morphine+vehicle, as well as, groups that received 1.0 mg/kg naloxone and then one of four doses of morphine on Day 2. The co-administration of 1.0 mg/kg naloxone blocked the significant reduction in contextual freezing that was
observed in morphine +vehicle treated groups. Significance is denoted by * $p<0.05$, **$p<0.01$, and *** $p<0.001$.

Figure 35. Morphine+ Naloxone Extinction of Contextual Fear.

This figure illustrates the percent conditioned freezing that was measured on Day 3 in groups which received morphine+vehicle, as well as, groups that received 1.0 mg/kg naloxone and then one of four doses of morphine on Day 2. The co-administration of 1.0 mg/kg naloxone blocked the significant facilitation of the extinction of contextual freezing that was observed in morphine +vehicle treated groups on Day 3. Significance is denoted by * $p<0.05$, **$p<0.01$, and *** $p<0.001$. 
Figure 36. Morphine + Naloxone Activity Data.

This figure illustrates the blocked increase in activity level measured on Day 2 conditioning in morphine + naloxone 1 mg/kg treated groups verses vehicle treated groups. Significance is denoted by * $p<0.05$, **$p<0.01$, and *** $p<0.001$.

**Mixed MOR agonist/ KOR antagonist contextual fear.** The semi-synthetic opioid buprenorphine, which exhibits partial agonism at MOR and antagonism at KOR, was tested. Buprenorphine significantly reduced freezing on Day 2, $F(3,28)= 3.774, p<0.05$, at all three doses tested 0.3 mg/kg, 1 mg/kg, and 3 mg/kg, $p<0.0001$ (Figure 37). Buprenorphine did not significantly facilitate extinction shown on Day 3 at any of the three doses, $p>0.05$ (Figure 37). Buprenorphine also significantly increased motion index scores over vehicle on Day 2 $F(3,28)= 8.936, p<0.0001$ at all doses tested, 0.3 mg/kg & 1 mg/kg, $p<0.0001$ and 3 mg/kg, $p<0.05$ (Figure 38).
Figure 37. Buprenorphine Contextual Fear.

This figure illustrates the significant reduction in percent contextual conditioned freezing on Day 2 in buprenorphine treated groups (0.3, 1 & 3 mg/kg) when compared to vehicle. Also shown is the lack of effect of buprenorphine on extinction of contextual conditioned freezing when compared to vehicle treated groups on Day 3. Significance is denoted by * $p<0.05$, **$p<0.01$, and *** $p<0.001$.

Figure 38. Buprenorphine Activity Data.
This figure illustrates the significant increase in activity levels that was measured on Day 2 conditioning in buprenorphine treated groups verses vehicle treated groups. Significance is denoted by * \( p<0.05 \), ** \( p<0.01 \), and *** \( p<0.001 \).

A summary table of the results from both experiments and their findings can be found below in Table 2. Results are grouped according to the type of ligand and their effects on the acute expression of freezing behavior, extinction, and whether these effects were stable at different times points tested in the time course for each drug.

<table>
<thead>
<tr>
<th>Type of Ligand</th>
<th>Acute Expression</th>
<th>Extinction Effects</th>
<th>Time Course</th>
</tr>
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<tbody>
<tr>
<td>KOR agonist</td>
<td>↑ Freezing</td>
<td>↑ facilitates</td>
<td>Stable</td>
</tr>
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<td>↑ facilitates</td>
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<td>MOR agonist/KOR antagonist</td>
<td>↓ Freezing</td>
<td>↔ No effect</td>
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</tbody>
</table>

**Discussion Experiment II**

**KOR modulation of fear behavior and extinction.**

Once the methods had been reworked with a concentration on contextual fear behavior and had the added measurement of extinction, I wanted to retest the KOR compounds, enadoline and U50,488. This was both for a direct comparison check on the new procedure but also because the Cain article used an anxiogenic drug to facilitate extinction of fear conditioning. Our
previous results showed a significant exacerbation of freezing with both enadoline and U50,488 but the effects they would have on extinction had not yet been explored.

The KOR agonist enadoline was tested and in the new procedure significantly exacerbated freezing in the conditioning context at the highest two doses (Fig 17) identical to the original contextual measurements from the first methods. These two highest doses also significantly reduced the motion index (Fig 18), mirroring the results of the original methods when total distance traveled was the variable measured. However, the interesting results are the Day 3 extinction tests which show that the highest dose of enadoline facilitated the extinction of freezing in the conditioned context (Fig 17). This follows the results that Cain (2004) observed using yohimbine an anxiogenic alpha 2 receptor antagonist. That two different receptor systems can produce the same effects in a similar task is extremely interesting. It also seems counterintuitive that an anxiogenic would improve extinction learning in an aversive task.

The KOR agonist U50,488 showed similar exacerbation of freezing in the conditioning context in the original methods but again extinction effects had not yet been measured. When tested using the new methods, U50,488 did not significantly exacerbate freezing in the conditioning context (Fig 19). This was interesting because the highest and lowest doses tested both significantly reduced motion index scores (Fig 20). I think that this shows that the motion index is sensitive to changes in activity that are not related to increases in freezing. Though U50,488 did not exacerbate freezing on Day 2, all doses tested facilitated the extinction of freezing in the conditioning context. I think that this shows that the kappa activation does not necessarily need to increase freezing to be effective as an aid to extinction learning.

The KOR antagonist norBNI was also tested using the new methods. As expected the compound showed anxiolytic properties and decreased freezing to the conditioning context at all doses tested (Fig 21). The highest dose tested also significantly increased motion index scores
Interestingly, the highest dose of norBNI significantly facilitated the extinction of freezing to the conditioning context (Fig 21). This suggests that the fluctuation of motion scores on Day 2 (either increased or decreased) is not predictive of the facilitation of extinction on Day 3. It is also intriguing that drugs with opposite actions at the same receptor can both produce similar effects on extinction. For further clarification of these effects it was important to establish if the extinctions effects we observed were stable over time.

The highest dose of enadoline was tested at different time points post extinction exposure to see if the facilitation of extinction was stable. Groups were tested at either Day 3, 7, or 14 after undergoing the same conditioning as before (Fig 23). The significantly facilitated extinction of freezing to the conditioning context with enadoline 0.1 mg/kg was the same no matter which day extinction was tested. All days the enadoline groups maintained a significantly lower percent freezing than vehicle groups, indicating that this facilitation of extinction was a stable phenomenon.

The highest dose of norBNI was tested at different time points post extinction exposure to see if the facilitation of extinction was stable. Groups were tested at either Day 3, 7, 14, 21, or 28 after undergoing the same conditioning as before (Fig 24). The significantly facilitated extinction of freezing to the conditioning context with norBNI 30 mg/kg was the same no matter which day extinction was tested. All days the norBNI groups maintained a significantly lower percent freezing than vehicle groups, indicating that this facilitation of extinction was also a stable phenomenon.

Since drugs with opposite receptor activity were both facilitating extinction it was of interest to see if the antagonist could block the agonist’s influence on this behavior. The administration of 10 mg/kg norBNI in conjunction with a full dose curve of enadoline was tested. This administration of the antagonist (administered 24 hours previous to enadoline due to
norBNI’s unique pharmacology) blocked all significant exacerbation of acute freezing to the conditioned context (Fig 25). Additionally the all significant motion index decreases by enadoline were blocked by norBNI (Fig27). Day 3 data on extinction are harder to interpret. The drug norBNI is active for at least three weeks days (Knoll & Carlezon, 2010), so the apparent blocking of the facilitation of extinction by enadoline may be due to norBNI’s continued action in vivo. The antagonist’s ability to block enadoline’s effects on fear behavior supports that this modulation is happening centrally.

**MOR modulation of fear behavior and extinction.**

The MOR analgesics fentanyl and morphine were both tested to evaluate their effects on acute fear behavior and the extinction of fear behavior. The analgesic fentanyl is an opioid agonist and is slightly more selective for the mu type receptor than morphine though their efficacy is similar (Volpe et al., 2011). Fentanyl significantly reduced freezing to the conditioning context on Day 2 at the intermediate dose (Fig 28). This dose also significantly increased the motion index on Day 2 (Fig 29). All three doses of fentanyl facilitated extinction of freezing to the conditioning context (Fig 28).

The MOR analgesic morphine was also tested and is of greatest interest due to the initiation of this project by clinical studies involving morphine’s apparent beneficial effects on trauma patients. Morphine significantly reduced freezing acutely at the two highest doses tested (Fig 30) while significantly increasing the motion index only at the highest dose tested (Fig 31). This could translate clinically to the acute relief of symptoms. Morphine also significantly facilitated the extinction of freezing to the conditioning context (Fig 30). Activation of the MOR appears to have beneficial effects both on the acute expression of fear behavior and on the facilitation of extinction.
The administration of the opioid antagonist naloxone was predicted to not reduce acute fear or affect the extinction learning process. When tested for its effects on freezing to the conditioned context naloxone had produced no significant increase or decrease on freezing behavior (Fig 32). Additionally naloxone did not significantly reduce or increase the effectiveness of extinction on Day 3 (Fig 32). Locomotor effects of naloxone were no different than that of vehicle. Naloxone administration didn’t produce any measurable effects on the expression or extinction of contextual freezing behavior in this assay.

The beneficial effects of morphine on the extinction and expression of conditioned freezing if produced by activation of mu receptors centrally should be blocked by the co-administration of naloxone. Naloxone (1 mg/kg) was administered in conjunction with a full dose curve of morphine. Co-administration of naloxone blocked the significant reduction of freezing to the conditioned context produced previously by morphine (Fig 4) on Day 2. Additionally, naloxone co-administration on Day 2 blocked the facilitation of the extinction of contextual freezing observed previously in the 1, 3, and 10 mg/kg morphine groups (Fig 35). The significant increase in motion index scores on Day 2 produced by the highest dose of morphine was also blocked by naloxone (Fig 36).

**Mixed MOR agonist/KOR antagonist modulation of fear behavior and extinction.**

The previous results suggest that the activation of MOR in conjunction with the antagonism of KOR might have beneficial effects on conditioned contextual freezing. The drug buprenorphine is a partial agonist at MOR and an antagonist at KOR, and is used clinically as a replacement treatment for opioid addiction as well as for chronic and acute pain (Howland, 2010). Buprenorphine was tested to ascertain its effects on freezing to the conditioned context. There was a significant decrease in contextual freezing on Day 2 at all doses of buprenorphine tested (Fig 37). Motion index was significantly increased for Day 2 at all doses tested (Fig 38).
but there was no facilitation of extinction by buprenorphine (Fig 37). The large increase in motion index makes data interpretation difficult as the declines in freezing may be due solely to locomotor activation effects. Regardless of Day 2 interpretation, no lasting changes were observed in freezing behavior on Day 3.

**General Discussion and Conclusions**

Overall, between the two experiments, there is evidence that opioid compounds can influence the expression and extinction of conditioned fear in C57BL/6J mice. In Experiment I it was observed that KOR agonists acutely exacerbated conditioned freezing in cue and contextual tests. There were some reductions in locomotor activity in the open field with kappa agonists and some long lasting increases in anxiety like locomotor behavior. It is unclear from the results of Experiment I if that change was solely due to KOR modulation, or was due to fear conditioning as similar changes were seen in the KOR antagonist groups. KOR antagonist norBNI reduced conditioned freezing acutely, and had no acute reduction in locomotor activity though as mentioned previously, it did result in the same pro-anxiety reallocation of behavior away from the center of the open field. These results supported previous research indicating that KOR antagonists display anxiolytic properties (Beardsley, Pollard, Howard, & Carroll, 2010; Carey, Lyons, Shay, Dunton, & McLaughlin, 2009; Schindler, Li, & Chavkin, 2010; Sperling, Gomes, Sypek, Carey, & McLaughlin, 2010) and that KOR agonists display anxiogenic properties (Carey, et al., 2009; Lemos, Roth, & Chavkin, 2011; Pezze & Feldon, 2004; Ponnusamy, Nissim, & Barad, 2005; Schindler, et al., 2010; Sperling, et al., 2010).

The conflicting results seen in the open field data showing that both compounds appeared to induce anxiogenic behavior could reflect a limitation of this procedure in predicting anxiolytic effects (Prut & Belzung, 2003), or could have been confounded by the exposure of the mice to the fear conditioning paradigm before exposure to the open field. One approach to
addressing the possible confound would have been to test additional groups of animals in the open field with just drug exposure instead of drug and fear learning exposure. Another method to counteract this confound was to use the movement index calculations generated within the fear conditioning chamber at the time of exposure as the measure of activity.

Additionally, in Experiment I diazepam, a positive allosteric modulator of GABA$_A$ receptors, was shown to also increase conditioned freezing and at the highest dose reduced locomotor activity in the open field. Initially, it was assumed that diazepam would serve as a positive control in this experiment as it is used as an anxiolytic clinically. Unfortunately, diazepam did not produce the expected anxiolytic like behavior in this assay. One explanation for this may have been that the majority of source material on diazepam in anxiety assays is in rats not mice (Asth, Lobao-Soares, Andre, Soares, & Gavioli, 2012; Shikanai et al., 2010; Zbinden & Randall, 1967). The previous research in mice exploring the effects of diazepam is mostly in other anxiety assays, elevated plus maze, light dark box, passive avoidance (Crestani, Assandri, Tauber, Martin, & Rudolph, 2002; Crestani et al., 2002; Pamplona et al., 2011). The effects of diazepam in other anxiety behavior assays is as predicted, but in fear conditioning the data are less consistent or diazepam is given prior to consolidation, not post training (Crestani, Assandri, et al., 2002; Crestani, Keist, et al., 2002; Pamplona, et al., 2011). Mice undergoing trace fear conditioning, for example, display enhanced freezing when given diazepam (Crestani, Keist, et al., 2002). This leads me to conclude that diazepam in this assay did not display anxiolytic properties. This left me without a positive control in this model, but this reflects the lack of a “gold standard” in clinical treatment for this disorder. The two approved treatments for PTSD as discussed previously are SSRI’s that do not produce anxiolytic effects with acute treatment. Therefore, it was decided to proceed with additional testing without this control, and interpret the obtained data based solely on the differences relative to vehicle conditions.
In Experiment II the same general results were observed for KOR agonists (increased conditioned contextual freezing) and KOR antagonists (decreased conditioned contextual freezing) acutely. In the newly obtained extinction data, however, it was observed that both KOR agonists and KOR antagonists facilitated extinction to conditioned contextual freezing. When a time course experiment was conducted, both the KOR agonist and the KOR antagonist facilitation of extinction were stable over several weeks with freezing levels staying at near baseline levels. When the KOR agonist (enadoline) and antagonist (norBNI) were co-administered, no increase in conditioned freezing was observed on Day 2. The KOR antagonist’s blocking of the acute exacerbation of freezing suggests that behavioral response is KOR mediated. However, Day 3 extinction data are difficult to interpret due to the long lasting effects of norBNI (Endoh, et al., 1992; Knoll & Carlezon, 2010). The facilitation of extinction by enadoline appears to be blocked by norBNI, but since norBNI is still pharmacologically active (Knoll & Carlezon, 2010) the level of conditioned freezing on Day 3 may reflect the continued antagonism of KOR.

Possible pathways for KOR modulation of fear conditioning.

The KOR in humans is distributed throughout the central nervous system and in peripheral tissues (Peng, Sarkar, & Chang, 2012). The highest concentration of KOR is in the putamen followed by the nucleus accumbens and caudate nucleus. While a moderate amount of KORs are also found in the hippocampus, substantia nigra, and dorsal root ganglion (Peng, et al., 2012). The endogenous ligands for KOR are the opioid peptides dynorphin A, dynorphin B, and α/β-neo-endorphin.(Day et al., 1998; Goldstein, Tachibana, Lowney, Hunkapiller, & Hood, 1979) which activate the both subtypes of the KOR 1 & 2 (Nyberg & Hallberg, 2007). Kappa opioid receptors are g-protein (G_i/G_o) coupled receptors. When activated by either endogenous ligands or exogenous ligands (e.g., dynorphin, enadoline, U50,488), there is a subsequent...
increase in phosphodiesterase activity. Since phosphodiesterases break down cAMP, this produces an inhibitory effect on neurons (Konkoy & Childers, 1993; Lawrence & Bidlack, 1993; Schoffelmeer et al., 1988). However, KORs also couple to inward-rectifier potassium and to N-type calcium ion channels (Henry, Grandy, Lester, Davidson, & Chavkin, 1995; Tallent, Dichter, Bell, & Reisine, 1994). Recent studies have also demonstrated that agonist-induced stimulation of the KOR, like other G-protein coupled receptors, can result in the activation of mitogen-activated protein kinases (MAPK). These include extracellular signal-regulated kinase, p38 MAP kinases, and c-Jun N-terminal kinases (Belcheva et al., 2005; Bohn, Belcheva, & Coscia, 2000; Bruchas, Macey, Lowe, & Chavkin, 2006; Bruchas, Xu, & Chavkin, 2008; Bruchas, Yang, et al., 2007; Kam, Chan, & Wong, 2004).

Stress has been shown to result in the release of many neuropeptides, among them dynorphin (Lemos, et al., 2011). One type of stress in mice that has been linked to the increase in release of dynorphins is forced swim stress. Mice that underwent forced swim showed activation of both KORs and p38 MAP kinase co-expressed in GABAergic neurons in the nucleus accumbens, cortex, and hippocampus; furthermore, this activation was KOR dependant as KOR knockout mice or wild type mice treated with norBNI did not show this activation (Bruchas, Land, et al., 2007). The activation of KORs, in mice, by exposure to a stressor like forced swim or by administration of a KOR agonist, has been shown to potentiate the reinforcing effects of drugs of abuse in behavioral assays like conditioned place preference, intracranial self stimulation and ethanol (Carey, et al., 2009; Schindler, et al., 2010; Sperling, et al., 2010).

Administration of the KOR antagonist norBNI blocks these stress-induced increases and KOR activation (Beardsley, et al., 2010). Genetic knockout of the KOR also blocks the stress-induced increases in these behaviors (Carey, et al., 2009; Schindler, et al., 2010; Sperling, et al., 2010).
These observations show a definite link between activation of the KOR and stress as well as a demonstrating that a blockade of the KOR can attenuate this effect.

Short term stress exposure in humans and rodents has long been observed to result in an increase in DA release in brain areas including the mesolimbic pathway and the nucleus accumbens (NAc) (Abercrombie, Keefe, DiFrischia, & Zigmond, 1989). Intense or chronic exposure to stress results in a decrease in DA in those same brain areas (Jensen et al., 2003; Marinelli et al., 2007). Stress and KOR activation show similar behavioral responses in the above mentioned assays and the activation of KOR has been shown to reduce DA in NAc (Pezze & Feldon, 2004; Ponnusamy, et al., 2005). Systemic administration of the KOR agonist salvonorin A reduces DA release in the NAc (Ebner, Roitman, Potter, Rachlin, & Chartoff, 2010) as does the KOR agonist U50,488 (Di Chiara & Imperato, 1988). The inhibition of dopamine transmission reduces conditioned fear (Pezze & Feldon, 2004). The systemic administration of both sulpiride, a dopamine₂ (DA) receptor antagonist and the antipsychotic clozapine (a DA₂ antagonist as well as other actions) facilitate extinction of conditioned fear (Jay et al., 2004; Ponnusamy, et al., 2005), while the administration of quinpirole (a DA₂ agonist) partially blocks extinction (Nader & LeDoux, 1999). This seems to indicate that a reduction of DA within the NAc results in an increased efficiency in extinction learning whether induced by a KOR agonist indirectly or direct DA₂ antagonism. In patients with PTSD a single photon emission computerized tomography (SPECT) imaging study revealed brain activity in the NAc was found to be higher than in controls (Liberzon et al., 1999). The reduction in DA in this brain region suggests that KOR agonists might be returning this brain area to a more normal level of activity though it is unknown if mice have an increase in basal levels of NAc activity post fear conditioning.
Additionally, changes in the DA levels in the NAc core versus the NAc shell have been shown to modulate memory consolidation (LaLumiere, Nawar, & McGaugh, 2005; Stevenson, Sullivan, & Gratton, 2003), and so the changes in this brain area could be improving the memory consolidation of extinction learning, though the acute effect is one of increased conditioned freezing. However, KOR activation in other learning models like novel object recognition is not effective in facilitating learning (Schindler, et al., 2010).

The mechanism by which KOR antagonists produce their long acting effects has been investigated and one hypothesis attributes it to stimulating c-Jun N-terminal kinase (JNK) phosphorylation (Bruchas, Yang, et al., 2007). Pretreatment of mice with the JNK inhibitor SP600125 before norBNI attenuates the long acting antagonism. The phosphorylation of JNK results in the functional disruption of KOR signaling (Bruchas, Yang, et al., 2007). KOR antagonists exhibit an overall anxiolytic and antidepressive profile in many preclinical assays (Beardsley, et al., 2005; Knoll, Meloni, Thomas, Carroll, & Carlezon, 2007). When tested in rats, KOR antagonists norBNI and JDTic dose-dependently increase open arm exploration in the Elevated Plus Maze without affecting Open Field behavior. They both also decreased conditioned fear in the Fear Potentiated Startle paradigm (Knoll, et al., 2007). This may indicate that KOR antagonists may be particularly effective for the treatment of comorbid depressive and anxiety disorders (Knoll, et al., 2007). The long term blockade of the KOR activation pathway results in the lack of activation of downstream KOR targets like extracellular signal-regulated kinase, p38 MAP kinases, and c-Jun N-terminal kinases (Belcheva, et al., 2005; Bohn, et al., 2000; Bruchas, et al., 2006; Bruchas, et al., 2008; Bruchas, Yang, et al., 2007; Kam, et al., 2004). Administration of KOR agonists in humans is reported to be aversive and depressive (Mizrahi et al., 2007). As mentioned above, KOR activation the NAc reduces dopamine function, which is associated with depressive and aversive effects in rodents (Nestler & Carlezon, 2006) though we
saw that it can also result in facilitation of extinction. It is possible that KOR antagonists make stress less aversive by counteracting the intracellular signaling cascades that regulate dynorphin expression (McLaughlin, Marton-Popovici, & Chavkin, 2003; Pliakas et al., 2001).

Possible pathways for MOR modulation of fear conditioning.

As previously mentioned, MOR agonist analgesics (i.e., morphine and fentanyl) are amongst the most common clinically prescribed pain relievers. They act through MOR activation, and MOR are located in diverse areas of both the CNS and PNS (Peng, et al., 2012). The highest concentration of MORs are located in the cerebellum, NAc, caudate nucleus, putamen, cortex and dorsal root ganglion (Peng, et al., 2012). Not only are these drugs used as pain relievers, but are used and abused not only among the general population but among PTSD patients. One study of US armed forces service members found that those with mental disorders were 2.5 more times as likely to be prescribed opioids, twice as likely to receive two or more concurrent prescriptions for opioids and a third more likely to seek early refills on opioid prescriptions (Seal et al., 2012). A previous study from the same group found that 11% of veterans of Iraq and Afghanistan conflicts met the criteria for substance abuse disorder and of those up to 75% received a concurrent diagnosis of depression or PTSD (Seal et al., 2011).

It is no surprise that opioids might possibly be used to alleviate anxiety. Many studies have demonstrated that during morphine withdrawal humans display anxiety and depression-related behaviors and there are corresponding behavioral responses in animals (Anraku, Ikegaya, Matsuki, & Nishiyama, 2001; Rezayof, Hosseini, & Zarrindast, 2009). Anxiety and depression associated with morphine withdrawal can be alleviated by the administration of antidepressant or anxiolytic drugs, such as fluoxetine (Zomkowski, Santos, & Rodrigues, 2005). In a preclinical model of anxiety, the elevated plus maze, pretreatment with morphine attenuates the restraint stress induced reductions in open arm entries and time spent in the open arms as compared to
vehicle treated controls (Anand, Gulati, & Ray, 2012). Rats experiencing persistent inflammatory pain induced by intraplantar injection of complete Freund's adjuvant show an anxiety phenotype in the elevated plus maze and the open field. This anxiety phenotype is reversed when rats were treated with morphine (Parent et al., 2012). Heroin addicted individuals, in comparison with healthy volunteers, exhibit significantly lower levels of adrenocorticotropic hormone, as well as have reduced levels HPA axis activation in response to a stressor (Gerra, et al., 2004; Ho, et al., 1977). Naloxone, an opiate receptor antagonist, increases HPA axis activity by blocking an inhibitory opioderigic influence on hypothalamic CRF secretion, and patients with PTSD have been reported to exhibit an exaggerated HPA axis response to naloxone. Interestingly, naloxone also has been shown to reverse the analgesia of PTSD patients after exposure to traumatic reminders. Also, PTSD patients exhibit increased CSF β-endorphin levels, suggesting increased activation of the endogenous opioid system. The opiate receptor antagonist, naltrexone, has been reported to be effective in treating symptoms of dissociation and flashbacks in traumatized patients (Newport & Nemeroff, 2000; Strawn & Geracioti, 2008). Morphine pretreatment attenuated stress induced release of NE in the thalamus, hypothalamus, hippocampus, amygdala and midbrain in rats subjected to restraint stress (Tanaka, et al., 1983).

Stress is considered a contributing factor in the vulnerability to opiate abuse and can play a role in initiating relapse in subjects with a history of abuse (Gaal & Molnar, 1990; Goeders, 1998, 2003; Hyman, Fox, Hong, Doebrick, & Sinha, 2007; Ilgen, Jain, Kim, & Trafton, 2008). Previous research also indicates that stress can alter individual sensitivity to opiates as well as suggesting that stress influences the synthesis and effectiveness of clinically used opiates (Benedek & Szikszay, 1985; Christie & Chesher, 1982; Christie, Trisdikoon, & Chesher, 1982; Sinha, 2001; Sinha, Catapano, & O'Malley, 1999; Sinha, Kinnerling, Doebrick, & Kosten, 2007; Stohr et al., 1999; Sutton, Grahn, Wiertelak, Watkins, & Maier, 1997). Conversely, long
term use of opiates can affect HPA axis responsiveness to stress and induce a greater individual sensitivity to stress-related psychiatric disorders (Burnett, Scott, Weaver, Medbak, & Dinan, 1999; Calogero et al., 1996; Carey, et al., 2009; Price, Risk, Haden, Lewis, & Spitznagel, 2004; Yamauchi, Shibasaki, Wakabayashi, & Demura, 1997). These known interactions between opioids and stress suggest that the effects we see in this model strengthens the theory that opioids influence and are influenced by stress.

One brain area that is of specific interest in the interaction of opiates and stress is the locus ceruleus, the major brain norepinephrine-containing nucleus. Many opioidergic peptides, including MOR agonists and antagonists, are known to act in the locus ceruleus (Kreibich et al., 2008; Reyes, Chavkin, & van Bockstaele, 2009; Reyes, Drolet, & Van Bockstaele, 2008; Reyes, Glaser, Magtoto, & Van Bockstaele, 2006; Reyes, Johnson, Glaser, Commons, & Van Bockstaele, 2007; Tjoumakaris, Rudoy, Peoples, Valentino, & Van Bockstaele, 2003; Van Bockstaele, Branchereau, & Pickel, 1995; van Bockstaele, Colago, & Pickel, 1996). The locus ceruleus is activated during stress exposure and opiates can influence this response (Valentino, Foote, & Page, 1993; Valentino & Wehby, 1988a). Chronic opiate use (Aghajanian & Wang, 1987; Duman, Tallman, & Nestler, 1988; Fiorillo & Williams, 1996; Valentino & Wehby, 1989) and chronic stress (Cuadra, Zurita, Lacerra, & Molina, 1999; Curtis, Pascovich, Grigoriadis, & Valentino, 1995; Curtis, Pascovich, & Valentino, 1999), chronic CRF (Conti & Foote, 1995, 1996) have been shown to induce changes in LC plasticity.

Environmental stimuli provoke phasic reactions of locus ceruleus neurons, which is associated with enhanced NE release in target regions (Berridge & Abercrombie, 1999; Florin-Lechner, Druhan, Aston-Jones, & Valentino, 1996). Opiates can also change the firing of the locus ceruleus (Bremner, et al., 1996).
In addition to arousal, the locus ceruleus-NE system is hypothesized to facilitate shifts in type of attention, from focused to scanning. This is supported by locus ceruleus recordings in nonhuman primates during a focused attention task (Aston-Jones, Rajkowski, & Cohen, 1999; Rajkowski, Kubiak, & Aston-Jones, 1994; Usher, Cohen, Servan-Schreiber, Rajkowski, & Aston-Jones, 1999). Inattention, drowsiness and poor task performance are associated with low tonic locus ceruleus discharge rate. Conversely, focused attention and optimal behavioral performance are associated with higher tonic locus ceruleus discharge rates, coupled with robust phasic responses to stimuli. If the increase tonic discharge rates are too excessive, then there is a reduction in attention to the target stimuli and poor task performance (Aston-Jones, et al., 1999; Rajkowski, et al., 1994; Usher, et al., 1999), which may indicate an inverted U-shaped relationship between tonic locus ceruleus activity and focused attention. This could be important when considering learning in response to environmental stimuli including stressful ones.

There is evidence of opioid receptor localization in the locus ceruleus and electrophysiological data showing opiate influence on locus ceruleus activity. The three classes of opioid receptors, MOR, DOR and KOR are prominently distributed within the LC (Van Bockstaele, et al., 1995; Van Bockstaele, Chan, & Biswas, 1996; Van Bockstaele, Chan, & Pickel, 1996). The MOR is localized postsynaptically within noradrenergic processes (Van Bockstaele, Chan, & Pickel, 1996; Van Bockstaele, Colago, Moriwaki, & Uhl, 1996), while the DORs and KORs are mainly localized on axon terminals (Kreibich, et al., 2008; Reyes, et al., 2009; van Bockstaele, Commons, & Pickel, 1997), this may mean that KOR and DOR have a role in the presynaptic release of neurotransmitters. Activation by endogenous or exogenous ligands of MOR on neurons in the locus coeruleus has an inhibitory effect in this region, and this is supported by in vivo and in vitro data (Aghajanian & Wang, 1987; Korf, Bunney, & Aghajanian,
The inhibitory action of MOR is linked to normal stress reactivity of the locus ceruleus because when stress exposure ends neurons there are transiently inhibited, this effect can be blocked by local micro-infusion of naloxone (Curtis, Bello, & Valentino, 2001). The local micro-infusion of naloxone into the locus ceruleus blocks activity of MOR. This blockade results in neuron activity in the locus ceruleus remaining elevated even though stress is over, which suggests that release of endogenous opioids might normally serve to return the activity of the locus ceruleus to normal levels (Curtis, et al., 2001). If put into terms of PTSD, continued high level of activity in the locus ceruleus could be reflected in the hyperarousal symptoms of the disorder. The release of endogenous opiates or the administration of exogenous opiates might serve to modulate the return of the locus ceruleus-NE system to normal function after exposure to stress has ended. When an individual is exposed to stress, CRF activates the locus ceruleus and attention is shifted from task oriented to scanning attention (Curtis, et al., 2001). This shift in attention can promote behavioral flexibility, but if shifted too far from baseline or for too long (after stress has ended) this can have a detrimental effect on cognitive processing (Curtis, et al., 2001). Data suggests that at the termination of stress endogenous opioids are released to inhibit the locus ceruleus system and return activity back to normal (Curtis, et al., 2001; Valentino, Page, & Curtis, 1991).

The data from my behavioral experiments show that both MOR and KOR ligands affect mouse conditioned freezing behavior. These results support the growing evidence that opioids are important compounds that influence stress behavior and should be further characterized due to their possible use as treatments in stress related disorders like PTSD, as well as, to understand stress related abuse consequences of opiates clinically.
Implications for the treatment of PTSD.

The data obtained in these preclinical experiments can be applied to our current clinical approach to PTSD treatment. The systemic administration of KOR agonists enadoline and U50,488 acutely exacerbated freezing behavior. However, if the KOR agonist administration took place shortly before an exposure extinction training session, then the extinction of freezing to the conditioned context was more effective than in animals treated with vehicle. If we apply this result to exposure therapy that is used with PTSD patients it suggests that there is a possibility that administration of a KOR agonist during exposure therapy could make this therapy more efficient as well as more effective. Exposure therapy is one of the most effective behavioral therapies in use with PTSD patients (Cooper, et al., 2005; Hetrick, et al., 2010). However, it is only successful in approximately 40% of patients. A second major concern which might contribute to this lack of efficacy is high dropout rates (~30%) during the lengthy treatment (8-15 weeks) (Cooper, et al., 2005; Hetrick, et al., 2010). It would then follow that if effective a KOR agonist might reduce the treatment length needed which may result in a higher therapy completion rate. An added benefit that might also be suggested by the data is that the administration of a KOR agonist might also make the therapy more effective regardless of effects on treatment length. The KOR antagonist norBNI produced reductions in acute conditioned freezing behavior as well as hastening extinction learning. So if applied to patients with PTSD that would mean that norBNI might provide acute symptom relief as well as improve the efficacy of exposure therapy. This would be a double benefit as current pharmacotherapies aren’t effective immediately. These data provide, further support for this protective relationship when we consider the current observations regarding MOR analgesics and their link to the reduced chances of developing PTSD. My data show that the administration of MOR agonists, morphine and fentanyl, shortly before measurement of acute conditioned freezing results in the significant
reduction in this behavior. This indicates that if applied to PTSD treatment the administration of MOR agonists might result in an acute reduction of some PTSD symptoms. This may help explain the increased rates of substance abuse in PTSD patients (for self-medication) and supports the use of MOR agonist analgesics in PTSD at risk populations. Additionally, the administration of MOR analgesics before exposure extinction training also produced more efficient extinction learning. The administration of MOR analgesics could function similarly as KOR agonists if used in conjunction with exposure therapy, but would also have the added benefit of acute symptom relief. The use of these MOR agonist analgesics, at or near the time of trauma, should have the added benefit of reducing later PTSD risk, and so it would be of interest to see if this holds true for non-injured PTSD at risk populations.

Finally, my results show that buprenorphine, a mixed MOR agonist/ KOR antagonist, might have a use as an adjunctive therapy in PTSD. While there seem to be no long term reductions in conditioned freezing, much like MOR analgesics or KOR antagonists, fast acute relief of some symptoms might be obtained using buprenorphine as an adjunctive treatment with SSRIs.

More study with opioid compounds is necessary before an understanding of their full impact on anxiety and posttraumatic stress disorder can be understood. So far however, it seems that the administration of opioids have generally beneficial effects on conditioned fear behavior. Most of the compounds investigated here are already used or at least have been studied in humans. The growing prevalence and lack of a “gold standard” treatment for posttraumatic stress disorder underline the importance of continuing to investigate the impact of opioids on this disorder and its treatment.
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Vita

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