# SUB-ANESTHETIC INTRAVENOUS KETAMINE INFUSION

## ON

## BEHAVIOR, BIOMARKERS, AND FEAR MEMORY

## IN

## MALE SPRAGUE-DAWLEY RATS

by

Kennett D. Radford II

Dissertation submitted to the Faculty of the
Daniel K. Inouye Graduate School of Nursing Doctor of Philosophy Program
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy 2017

Distribution Statement

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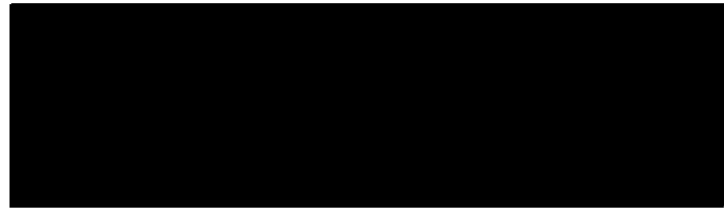Penny F. Pierce PhD, RN, FAAN

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Memory in Male Sprague-Dawley Rats.

Kennett D. Radford, Doctor of Philosophy, 2017

Dissertation directed by:

T. John Wu, PhD, Professor, Department of Obstetrics and Gynecology, Uniformed
Services University of the Health Sciences.

**Background:** Ketamine is a multimodal dissociative anesthetic and potent analgesic
administered to the combat wounded following traumatic injury. Since ketamine induces
transient psychosis effects, there are concerns regarding its impact on the development of stress
related disorders. There are a limited number of pre-clinical rodent investigations that have
examined the effects of post-fear conditioning ketamine on fear memory and PTSD-like
behaviors. However, there are no pre-clinical investigations that administered post-fear
conditioning ketamine via the intravenous (i.v.) route to rodents to measure outcomes on fear
memory processes.

**Methods:** We conducted a series of three experiments to address this gap. Our first objective
was to determine optimum sub-anesthetic i.v. ketamine dosages (bolus and infusion) in rats that
produced analgesia, while minimizing dissociative behaviors. We administered a sub-anesthetic
i.v. ketamine bolus (0, 2, and 5 mg/kg) and infusion (0, 5, 10, and 20 mg/kg/h) to male Sprague-
Dawley rats and measured outcomes in dissociative behaviors, locomotor activity, analgesia,
sensorimotor gating, and plasma drug concentration. For our next experiment, we administered
sub-anesthetic i.v. ketamine infusions after fear conditioning (0, 5, and 20 mg/kg/h) and after no

fear conditioning (0 and 20 mg/kg/h) and measured effects on plasma biomarkers of stress (corticosterone – CORT) and memory (brain-derived neurotrophic factor – BDNF). Our final experiment measured the effects of immediate (0, 1, 5, and 10 mg/kg/h) and 1-day delayed (0 and 5 mg/kg/h) post-fear conditioning ketamine infusions on activity and fear memory retrieval, fear memory extinction, and fear memory renewal.

**Results:** *Experiment 1:* There were dose-dependent changes in dissociative stereotypy at all dosages. Ketamine dosages of 5 mg/kg per bolus and 20 mg/kg/h per infusion increased locomotion, prolonged analgesia, and impaired sensorimotor gating, while 5 mg/kg/h suppressed locomotion. *Experiment 2:* Sub-anesthetic i.v. ketamine infusions (5 and 20 mg/kg/h) produced dose-dependent elevations in plasma CORT and reduced plasma BDNF. *Experiment 3:* Immediate and delayed ketamine 5 mg/kg/h infusions suppressed locomotor activity, increased fear memory retrieval, delayed fear extinction, and enhanced fear renewal. Immediate ketamine infusions (1 and 10 mg/kg/h) also increased fear memory retrieval but did not delay extinction or enhance fear renewal.

**Conclusions:** Overall, these data validate our rodent sub anesthetic i.v. ketamine infusion model and demonstrate dose-dependent changes to behavior, biomarkers of stress and memory, and fear memory behaviors. Our results suggest that a sub-anesthetic ketamine i.v. infusion administered after trauma is detrimental towards trauma and stress related disorders.

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| 2017 | Effect of intravenous ketamine on corticosterone and brain-derived neurotrophic factor in male Sprague-Dawley rats. | Tri-Service Nursing Research Program, Ellicott City, MD. |
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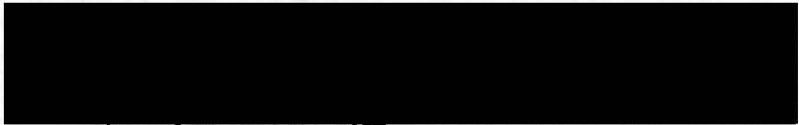| 2016 | The effect of intravenous ketamine on dissociation, locomotor activity, information processing, and antinociception in rat. | The Amygdala Conference, Uniformed Services University, Bethesda, MD. |

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# APPROVAL SHEET

Title of Dissertation:        Sub-anesthetic Intravenous Ketamine Infusion on Behavior, Biomarkers, and Fear Memory in Male Sprague-Dawley Rats.

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# DEDICATION

*For my mother. You left your fingerprints on my soul and I will never forget your memory.*

# ACKNOWLEDGEMENTS

"Alone we can do so little; together, we can do so much" – Helen Keller

I accomplished this doctoral journey with a strong, talented, and supportive team.

To my wife Heidi, you are a beautiful and caring woman with unlimited love. You are my eternal cheerleader and have always supported me as I pursued my dreams. Words cannot express the tremendous gratitude I have for all the sacrifices you have made in order to support my goals. This accomplishment is as much yours as it is mine! Thank you and I love you.

To my children, Kaylee and Adam, I love each of you immensely. Both of you have grown so much over the past three years while I've been working tirelessly on my research. I appreciate your forgiveness as I missed the occasional game or track meet – you each have such caring hearts. I can't thank you enough for giving me the time and space I needed to complete this milestone.

To Dr. Kwang Choi, you were instrumental in helping me complete this project. You took a chance and agreed to mentor me in your lab and for that, I am forever grateful. We spent countless hours conversing in the lab, over lunch, by email, and on the phone discussing our research. You patiently tolerated my non-stop questions while teaching me rodent behavioral research, basic bench science techniques, and manuscript preparation. You have been so kind to me and my family and I'm proud to call you my friend and mentor.

To Thomas Park, you were absolutely vital in completing this research as an assistant in Dr. Choi's lab. We spent countless hours in the lab completing various experiments. Our conversations allowed me to brainstorm and work through obstacles and I sincerely valued your "off-duty" time in which you helped me complete experiments, analyze data, and proof-read manuscripts. Thank you.

To Dr. Lisa Osborne-Smith, you served not only as my academic advisor but as my Navy mentor. You inspired the early direction of this research project and provided me advice and wisdom along my doctoral journey. Your keen insight into grant writing, research design, and publication have been instrumental to our team's success. Thank you so much for your scholarly and Navy guidance.

To my dissertation committee (Dr. T. John Wu, Dr. Lisa Osborne-Smith, Dr. Kwang Choi, and Dr. Danette Cruthirds), thank you for your patience. Each of you have volunteered your time to guide me over the past year. You patiently listened to my concerns or questions as I cornered you in hallways, offices, or even the cafeteria and provided me sound advice. I sincerely appreciate your time and helping me to push me forward.

To the faculty, thank you for sharing your knowledge. I can say, without a doubt, the faculty at the Uniformed Services University have been the best I have ever encountered. Each of you imparted knowledge, patiently answered questions, and inspired my pursuit to create knowledge and share it with others. Dr. Penny Pierce, Ph.D. Program Chair, thank you so much for your guidance, wisdom, and constant words of encouragement. When you spoke – I listened. Thank you.

To my fellow PhD students, thank you for our time together. I worked with students in both the Neuroscience Graduate Program and the Graduate School of Nursing. We studied hard, we laughed a lot, and we have many fond memories. We share a bond that will never be broken.

To the support staff, thank you for all of your work behind the scenes. Especially, Shohreh Razi and Sheilah Rowe, who are grant management specialists. There are too many others to name, but without the generous support from each of you, I could not submit grants,

obtain books, register for classes, complete official travel, print posters, provide care for my

animals, and so much more. You are the glue that held myself and my project together.

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Kennett D. Radford II

October 02, 2017

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Kennett D. Radford, Doctor of Philosophy, 2017

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**Methods:** We conducted a series of three experiments to address this gap. Our first objective was to determine optimum sub-anesthetic i.v. ketamine dosages (bolus and infusion) in rats that produced analgesia, while minimizing dissociative behaviors. We administered a sub-anesthetic i.v. ketamine bolus (0, 2, and 5 mg/kg) and infusion (0, 5, 10, and 20 mg/kg/h) to male Sprague-Dawley rats and measured outcomes in dissociative behaviors, locomotor activity, analgesia, sensorimotor gating, and plasma drug concentration. For our next experiment, we administered sub-anesthetic i.v. ketamine infusions after fear conditioning (0, 5, and 20 mg/kg/h) and after no

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**Results:** *Experiment 1:* There were dose-dependent changes in dissociative stereotypy at all dosages. Ketamine dosages of 5 mg/kg per bolus and 20 mg/kg/h per infusion increased locomotion, prolonged analgesia, and impaired sensorimotor gating, while 5 mg/kg/h suppressed locomotion. *Experiment 2:* Sub-anesthetic i.v. ketamine infusions (5 and 20 mg/kg/h) produced dose-dependent elevations in plasma CORT and reduced plasma BDNF. *Experiment 3:* Immediate and delayed ketamine 5 mg/kg/h infusions suppressed locomotor activity, increased fear memory retrieval, delayed fear extinction, and enhanced fear renewal. Immediate ketamine infusions (1 and 10 mg/kg/h) also increased fear memory retrieval but did not delay extinction or enhance fear renewal.

**Conclusions:** Overall, these data validate our rodent sub anesthetic i.v. ketamine infusion model and demonstrate dose-dependent changes to behavior, biomarkers of stress and memory, and fear memory behaviors. Our results suggest that a sub-anesthetic ketamine i.v. infusion administered after trauma is detrimental towards trauma and stress related disorders.

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| | |
|---|---|
| ACTH | adrenocorticotrophic factor |
| ASR | acoustic startle reflex |
| AMPA | alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid |
| AUC | area under the curve |
| BDNF | brain-derived neurotrophic factor |
| BLA | basolateral amygdala |
| CeA | central amygdala |
| CNS | central nervous system |
| CORT | corticosterone |
| CR | conditioned response |
| CS | conditioned stimulus |
| d | day |
| DSM-5 | Diagnostic and Statistical Manual of Mental Disorders, fifth edition |
| DoD | U.S. Department of Defense |
| FC | fear conditioning |
| FDA | Food and Drug Administration |
| GABA | $\gamma$-aminobutyric acid |
| h | hour |
| HPA | hypothalamic-pituitary-adrenal axis |
| IFS | inescapable footshock |
| IL | infralimbic |
| i.m. | intramuscular |
| i.v. | intravenous |
| i.p. | intraperitoneal |
| ITC | intercalated cells |
| ITI | inter-trial interval |
| kg | kilogram |
| LCMS | liquid chromatography/mass spectrometry |
| LH | lateral hypothalamus |
| LTP | long-term potentiation |
| PCP | phencyclidine |
| PL | prelimbic |
| PPI | pre-pulse inhibition |
| m | minute |
| MDD | major depressive disorder |
| mg | milligram |
| mL | milliliter |
| mPFC | medial prefrontal cortex |
| mRNA | messenger ribonucleic acid |
| msec | millisecond |
| mTOR | mammalian target of rapamycin |

| | |
|---|---|
| NIH | National Institutes of Health |
| NMDA | $N$-methyl-$D$-aspartate |
| mTBI | mild traumatic brain injury |
| PDG | periaqueductal grey |
| PFC | prefrontal cortex |
| PSS | predator scent stress |
| PVN | paraventricular nucleus |
| PTSD | post-traumatic stress disorder |
| SEM | standard error of the mean |
| s.c. | subcutaneous |
| US | unconditioned stimulus |
| U.S. | United States |
| WHO | World Health Organization |

# CHAPTER 1

## 1. Introduction

Wounded warfighters are surviving traumatic combat related injury at historic rates due to technological and medical advances but are twice as likely to develop post-traumatic stress disorder (PTSD) compared to those without physical injury [1, 2]. Forward deployed medical providers administer sedatives and analgesics to the traumatically wounded but the short and long-term impact of these agents on fear memory processes are not clear. Ketamine, a noncompetitive antagonist at the *N*-methyl-*D*-aspartate (NMDA) receptor, is a preferred battlefield anesthetic due to its potent analgesia, cardiopulmonary stability, and wide therapeutic window [3-8]. Although a seemingly ideal trauma analgesic, ketamine at sub-anesthetic dosing is also known to induce dissociation, hallucination, and delusions [9, 10], which may dysregulate fear memory processing. Dysfunctional fear memories that fail to extinguish lead to intrusive symptoms that can persist for months to decades following a traumatic event and result in increased psychological suffering and significant utilization of health care resources [11-16]. Therefore, understanding the effects of post-trauma ketamine administration on fear memory processes are the initial steps in addressing the larger impact of anesthetics and analgesics on trauma and stressor-related disorders.

## 2. Post-Traumatic Stress Disorder

### 2.1 Significance

PTSD is a debilitating stress-associated psychiatric condition that places a substantial burden on both the individual, their families, and society. It is estimated that 70% of the United States (U.S.) population experiences one or more exposures to a traumatic event in their lifetime, and that up to 6.8% will have been diagnosed with PTSD [17, 18]. In comparison, the lifetime

prevalence among U.S. veterans from the Iraq and Afghanistan wars is two- to four-fold higher than civilians with rates ranging from 14 – 24% [14, 19]. Veterans with PTSD are not only at greater risk for depression [20], substance use [21], and suicide [22], but their families also suffer. PTSD increases the likelihood for relationship conflict [23, 24], parenting difficulties [25], and interpersonal violence [26].

PTSD also negatively impacts the organization. The two-year post-deployment economic burden to the Department of Defense (DoD) for service members co-diagnosed with PTSD and major depressive disorder (MDD) is estimated at $6.2 billion, which is mostly attributed to loss of workplace productivity [27]. Additionally, the Veterans Health Administration spends roughly $8,000 (2011 dollars) for each veteran diagnosed with PTSD during their first year of care, which is second to traumatic brain injury [28]. Overall, PTSD is a significant health care concern for veterans, their families, and military leadership.

*2.2 Diagnosis*

Most trauma victims will experience a range of fearful emotions following a traumatic event but they will eventually adapt and recover, typically over a two to three week period [29]. However, those who suffer from PTSD continue to experience persistent, overgeneralized expressions of fear, more than a month, and up to decades following an event [15]. Categorized under "Trauma- and Stressor-Related Disorders," the fifth-edition of the American Psychiatric Association's *Diagnostic and Statistical Manual of Mental Disorders* (DSM-5) provides specific diagnostic criteria for PTSD [11]. First, an individual must be exposed to actual or threatened death, serious injury, or sexual violence through either direct experience, witnessing, repeated exposure to details, or learning of a traumatic occurrence of a close friend or family member. Next, the individual must experience one or more symptoms within the following four domains: intrusive symptoms, avoidance behavior, negative alteration of cognition and mood, and

hyperarousal. A key feature of PTSD is the intrusive symptom cluster, which manifests as recurrent and involuntary memories, traumatic nightmares, flashbacks, and prolonged psychological distress to trauma-associated cues. These intrusive symptoms of PTSD often result from maladaptive and dysregulated fear [30-32].

## 3. Fear

### 3.1 Definition

Fear is often misconstrued with the term anxiety, and although they are closely intertwined and share similar neurobiological circuits, they are different constructs. Anxiety is a generalized response to an internal struggle or an unknown external danger, whereas, fear is the response to a known and specific external threat [33]. Some have regarded fear as a distinctly human experience. For instance, Joseph LeDoux describes fear as a human subjective feeling or emotion that occurs after conscious appraisal of stimuli that threatens survival [30]. Hence, he cautions against applying the term fear, a human emotion, to organisms that cannot describe their conscious evaluation of an event. LeDoux prefers that researchers use the term 'threat' in place of 'fear' when describing animal processes. However, most ethologists have traditionally described fear as the motivational state of an organism that produces defensive or escape behavior in response to specific stimuli [33]. Thus, a greater emphasis is placed on observable behavior rather than a conscious assessment. It is not surprising then that the most prevalent use of 'fear' across animal behavioral literature is that of the traditional ethological description. Therefore, for the purposes of this document, (rodent) *fear* is operationally defined as the expression of no bodily movement (freezing) with the exception of respiration, in response to a threat-associated cue or context.

### 3.2 Fear Memory

Fear memory is a protective mechanism preserved across species as a means to remember threatening information and trigger fear behavior and physiologic responses during a future encounter to preserve life. Memories are created, stored, recalled, and degraded through a series of complex and overlapping stages of acquisition, consolidation, retrieval, and extinction [34-36]. Fear memories are primarily formed and regulated in the limbic system, which is comprised of the medial prefrontal cortex (mPFC), hippocampus, and amygdala [37, 38]. The amygdala is a key region involved in fear processing as it attaches emotional valence to a memory and regulates fear responses [39]. The hippocampus encodes contextual information regarding the environment [40], while the mPFC regulates top-down control of the amygdala and is instrumental in safety learning, also called fear extinction [41]. Disruptions to the activity and function of limbic brain regions associated with fear memory are associated with trauma and stressor related disorders [42-45].

*3.3 Animal Models*

There are ethical and technical challenges related to prospective studies of human fear memory and behaviors. Since the underlying brain circuits involved in fear processing are consistent across mammalian species, pre-clinical researchers use animal models as an alternative approach to study fear memory circuits, biomarkers, and PTSD-like behaviors [42, 46, 47]. Animal models of PTSD are generally classified into one of the following paradigms based upon the stressor type used to generate PTSD-like behaviors: physical stressors, social stressors, and psychological stressors (Table 1.1) [48]. Moreover, the stressor used in stress paradigms should meet five evaluation criteria [49]. A stressor should: (1) produce biological and behavioral effects of PTSD, (2) dose-dependently produce PTSD-like effects, (3) induce biological changes that persist over time, (4) have the potential for bidirectional expression, and (5) produce individual variance. There is no consensus among researchers regarding the ideal

animal model of PTSD. Each stress paradigm has advantages and disadvantages that researchers must consider.

| Physical | Social | Psychological |
|---|---|---|
| Footshock | Housing instability | Predator and predator odor |
| Stress-enhanced fear learning | Early life stress | |
| Underwater trauma | Social defeat | |
| Restraint Stress | | |
| Single prolonged stress | | |

Table 1.1. **Types of stressors used within animal models of PTSD.** Adapted from Whitaker, Gilpin, & Edwards (2014) [48].

*3.4 Footshock*

Footshock is a commonly used physical stressor in rodent PTSD models. It is generally administered through an electrically charged wire rod floor from which the rodent cannot escape. The footshock stressor meets 4 of the 5 criteria used to evaluate animal models of PTSD [48] with additional translational advantages. Inescapable footshock is a nociceptive stimulus that resembles an acute pain experience in humans. Researchers control the footshock intensity and duration to generate varying degrees of nociception that correspond to human trauma severity. Additionally, researchers administer footshocks at a discrete time point to rodents in order to mimic a singular traumatic event similar to a human trauma experience. Additionally, footshock is a well-established physical stressor that is frequently used as an aversive unconditioned stimulus in rodent fear conditioning investigations.

## 4. Fear Conditioning

*4.1 Overview*

Ivan Pavlov first described behavioral conditioning when he observed dog salivation in response to a ringing bell [50]. Since Pavlov, researchers have used various types of conditioning experiments to study learning and behavior. Fear conditioning is a form of associative learning frequently used to explore the neurobiological mechanisms of fear memory and behavioral responses to threat-associated cues [51]. In this model, investigators repeatedly pair a neutral conditioned stimulus (CS), such as an auditory tone or visible light with an aversive unconditioned stimulus (US) such as electrical footshock (Figure 1.1). This process conditions the rodent brain to learn or acquire a relationship between the CS and US, so that the CS signals an impending threat (i.e. footshock). Following acquisition, a memory undergoes consolidation where it is stabilized into a long-term memory. Therefore, the presentation of the CS at a future time will activate the CS-US association and induce a conditioned response (CR), such as freezing behavior. Similar to specific cues, the rodent also learns a relationship between the environment and US, such that exposure to the environment alone will induce threat anticipation and cause freezing behavior.
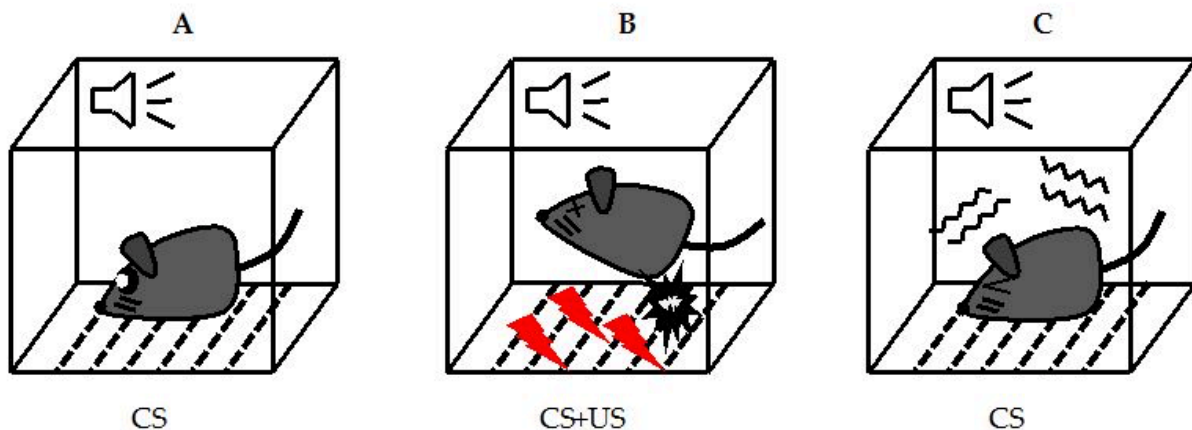


Figure 1.1 **Fear conditioning** A) The CS (conditioned stimulus; auditory tone) alone does not elicit freezing. B) The CS is paired with the US (unconditioned stimulus; footshock). C) A future presentation of the CS induces freezing as a conditioned response (CR). Figure adapted from [52].

*4.2 Face Validity*

Humans undergo fear conditioning in response to a traumatic event [53]. A wounded warfighter forms an association between pain (the US) and other cues such as sights, sounds, and smells (the CS) at the time of injury [54]. Exposure to similar CS cues or the trauma environment at a later time can trigger the intrusive and hyperarousal symptoms that characterize PTSD such as distressing memories, flashbacks, and intense physiologic responses (increased heart rate, blood pressure, cortisol) [55]. Therefore, the rodent fear conditioning model has significant face validity because it produces similar behavioral and physiological responses associated with stress related disorders [56]. These similarities across species have allowed researchers to use fear conditioning as a valid model to study fear memory circuitry within the brain [37, 56, 57].

## 5. Fear Memory Circuitry

*5.1 Hebbian Theory of Synaptic Plasticity*

The associative learning that occurs during fear conditioning is a form of long term potentiation (LTP). LTP is the strengthening of synapses between neurons, which is also referred to as synaptic plasticity [58]. Donald Hebb was the first to describe synaptic plasticity when he observed that repeated presynaptic neuron activation of a post synaptic neuron resulted in increased growth and efficiency in communication [59]. Hebb's description, now referred to as Hebbian Theory or Hebbian Plasticity, forms the underlying theoretical framework for learning and memory within brain circuits. The colloquial phrase coined by Dr. Carla Shatz, "cells that fire together, wire together," is a succinct and accurate summary of the plasticity phenomenon [60]. Normally, a sound stimulus alone is not strong enough to elicit a fear behavior response; however, when the weak sound stimulus (CS) repeatedly converges with a strong painful stimulus (US) on the same post-synaptic neuron, the co-occurring CS-US stimuli will strengthen

the synaptic connections between them and enhance neuronal communication and activation. Ultimately, these plastic changes allow the previously weak CS the new founded ability to trigger a CR, which is the freezing behavior observed in a fear conditioning model.

*5.2 Fear Memory Formation and Retrieval*

The amygdala is the principal site of fear memory formation and Hebbian plasticity. The painful footshock stimulus (the US) and the neutral auditory tone stimulus (the CS) converge onto post-synaptic neurons within the amygdala at a region called the basolateral amygdala complex (BLA) (Figure 1.2) [39]. Pre-synaptic neurons release glutamate, an excitatory neurotransmitter, that binds to post-synaptic NMDA receptors and trigger calcium entry into the cell [61]. Calcium initiates a series of second messenger systems and neurochemical cascades that reach the cell nucleus. The nucleus then drives the production of new proteins through the transcription of messenger ribonucleic acid (mRNA), which migrate into the cytoplasm where they are translated to proteins such as brain-derived neurotrophic factor (BDNF) [62]. BDNF increases dendritic and neuronal spine growth that leads to increased synaptic strength. The *de novo* protein synthesis within the BLA is essential for memory consolidation, which stabilizes a short-term memory to a long-term fear memory [63]. The length of the consolidation period in rodents and humans is not well understood but it is generally estimated to occur within the first few hours after fear conditioning [62, 64-66].

Enhanced consolidation within the amygdala may increase the risk for PTSD. The over-consolidation hypothesis states that exaggerated fear memory formation during the consolidation period may lead to the intrusive symptoms that characterize PTSD [67]. In support of this hypothesis, animal studies have shown that increased noradrenergic activity and acutely elevated corticosterone (CORT), a rodent stress hormone, within the amygdala after fear conditioning enhance memory consolidation [68, 69]. Moreover, human subjects diagnosed with PTSD show

increased amygdala activity compared to matched controls when presented with negative stimuli [70-72]. The results from both pre-clinical and human studies highlight the central role of the amygdala in the formation and retrieval of fear memory.

The hippocampus projects to the amygdala and encodes contextual information regarding the environment (Figure 1.2). Lesions applied to the hippocampus after fear conditioning do not affect the freezing response to the CS cues [73, 74]; however, hippocampal lesions disrupt freezing to the fear conditioning environment. These studies suggest a key role of the hippocampus to modulate the contextual information of fear memories within the amygdala [40]. Altered hippocampal function is implicated in PTSD. Animal studies have shown that stress and increased glucocorticoid exposure impairs dendritic growth within rodent hippocampal neurons [75]. Similarly, brain imaging of human subjects diagnosed with PTSD demonstrate reduced hippocampal volumes compared to matched controls [76-78]. Taken together, these studies demonstrate that impairment of hippocampal function leads to dysregulated fear memory recall and intrusive PTSD symptomology [79].
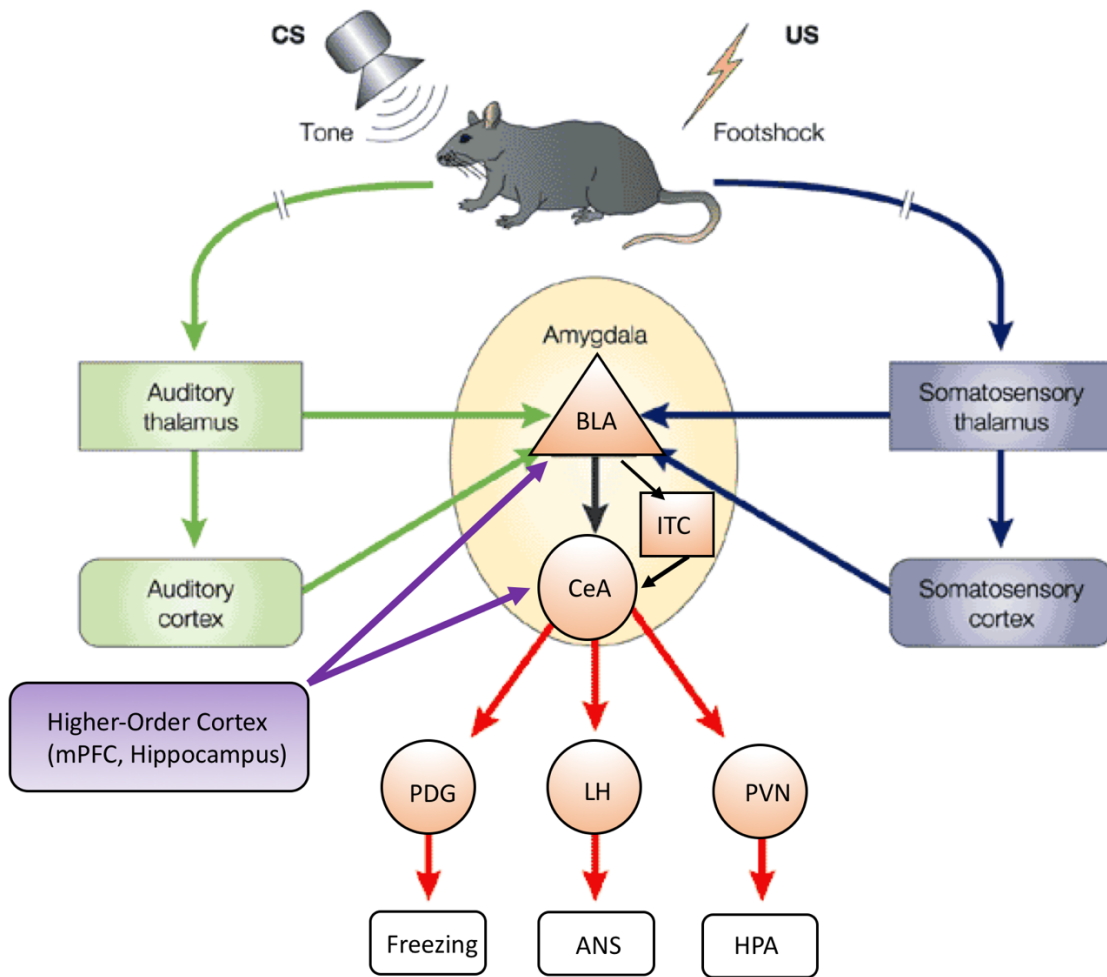
Figure 1.2 **Neural Circuits during fear conditioning.** CS and US stimuli converge onto the basolateral nucleus of

the amygdala (BLA). Information is then transmitted to the intercalated cells (ITC) and central nucleus of the

amygdala (CeA), which project to regions responsible for fear behaviors and physiologic responses (PDG:

periaqueductal grey area, LH: lateral hypothalamus, PVN: paraventricular nucleus of hypothalamus, ANS:

autonomic nervous system, HPA: hypothalamic-pituitary-adrenal axis). Higher-order cortex structures (mPFC:

medial prefrontal cortex and hippocampus) also regulate amygdala dependent fear conditioning and behavioral

response. Adapted from [80]

## 5.3 Fear Response

Although fear memory (the CS-US association) is formed and stored in the BLA complex

[39], the fear response output signals originate within a different region called the central nucleus

of the amygdala (CeA) (Figure 1.2). The presentation of the CS (auditory tone) to a previously fear conditioned rodent will activate the associative post-synaptic neurons in the BLA, which project to the CeA via two different pathways. The BLA communicates with the CeA directly or via the intercalated cells (ITC) (Figure 1.2), which serve as a bridge between the two regions [81]. The CeA then projects output signals that trigger behavioral and physiologic changes (Figure 1.2). Characteristic freezing behavior is regulated by signals from the periaqueductal gray (PDG) area [82, 83], while signaling from the lateral hypothalamus (LH) trigger ANS responses such as increased heart rate and blood pressure [83]. Activation of the paraventricular nucleus (PVN) of the hypothalamus, induces the hypothalamic-pituitary-adrenal (HPA) axis to release adrenocorticotrophic hormone (ACTH) from the pituitary and CORT from the adrenal cortex [84]. Although the CeA is the primary output center for physiologic and behavioral fear responses, it is regulated through higher-order cortical control (Figure 1.2).

The mPFC provides cortical regulatory control over amygdala driven fear responses. Interestingly, subregions of the mPFC serve to either enhance or suppress fear expression [85]. Activation of the prelimbic (PL) PFC subregion (ventromedial PFC in humans) is required to drive the fear response. Lesions applied to the PL reduce fear expression to context and cues in fear conditioning models, which support a necessary role for the PL in fear output [86]. Further work has shown that the PL integrates a variety of information from the amygdala, hippocampus, brainstem, and orbital and lateral PFC prior to signaling [87]. If an excitatory threshold is exceeded, the PL signals the amygdala (CeA) to initiate a fear response. On the other hand, the infralimbic (IL) PFC subregion suppresses fear behavior and regulates safety learning through fear extinction [85].

*5.4 Fear Extinction, Spontaneous Recovery, and Fear Renewal*

The rodent fear extinction paradigm is similar to exposure-based therapies used in humans to treat stress and anxiety related disorders. The underlying principal of exposure therapy is that a clinician will expose a patient to threat associated cues in a safe environment to erode the threat association and reduce PTSD symptoms. Similarly, rodent fear extinction training utilizes repeated presentations of a non-reinforced CS in a novel context that is different from the fear conditioning environment. The non-reinforced CS exposure gradually reduces the conditioned freezing behavior because the animal forms a safe (CS-no US) association that overrides the previous fear (CS-US) association (Figure 1.3A).

Fear extinction is primarily regulated through mPFC suppression of the amygdala. The mPFC integrates thalamic and hippocampal inputs [88] regarding sensory and environmental information. If determined as non-threatening, the infralimbic (IL) PFC subregion (dorsal anterior cingulate cortex in humans) projects inhibitory output signals to both the ITC and CeA amygdaloid regions [89, 90]. CeA inhibition then suppresses output to the hypothalamus and brain stem, which in turn, reduce fear behavior and fear associated physiologic responses. Similar to fear learning, fear extinction requires *de novo* protein synthesis in the mPFC [91] and amygdala [92], which serve to consolidate extinction training into a long-term memory [93]. However, mechanisms that impair the IL-CeA extinction circuitry could disrupt fear extinction learning and contribute towards PTSD-like behaviors [94, 95].

Fear extinction does not permanently reduce or erase fear memory because fear extinction is reversible. The most common examples of fear extinction reversal are spontaneous recovery and fear renewal. Spontaneous recovery is the partial return of freezing behavior with the passage of time after fear extinction (Figure 1.3B) [36]. The degree of spontaneous recovery is directly related to the elapsed time interval between extinction learning and CS re-exposure such that longer time intervals result in greater recovery of conditioned freezing behavior [96].

On the other hand, fear renewal is the reversal of fear extinction upon return to the fear conditioning context [36]. Extinction learning is highly context dependent due to hippocampal regulatory input to the IL. Typically, fear extinction learning occurs in a novel environment (context B) from fear conditioning (context A) (Figure 1.3A). Fear renewal (increased freezing behavior) is observed when a previously fear extinction trained rodent is returned to the fear conditioning context (Figure 1.3C).

Spontaneous recovery and fear renewal also translate to humans who have undergone exposure therapy for PTSD. For example, a service member diagnosed with combat-related PTSD who receives therapy in an office setting may experience both fear renewal and spontaneous recovery of PTSD symptoms upon returning to a similar combat environment at a later time. To address these context dependent phenomena, pre-clinical researchers may use an ABBA context design to measure treatment effects on fear conditioning (context A), fear extinction (context B), spontaneous recovery (context B), and fear renewal (context A).

## A. Extinction is not the same as forgetting



## B. Spontaneous recovery
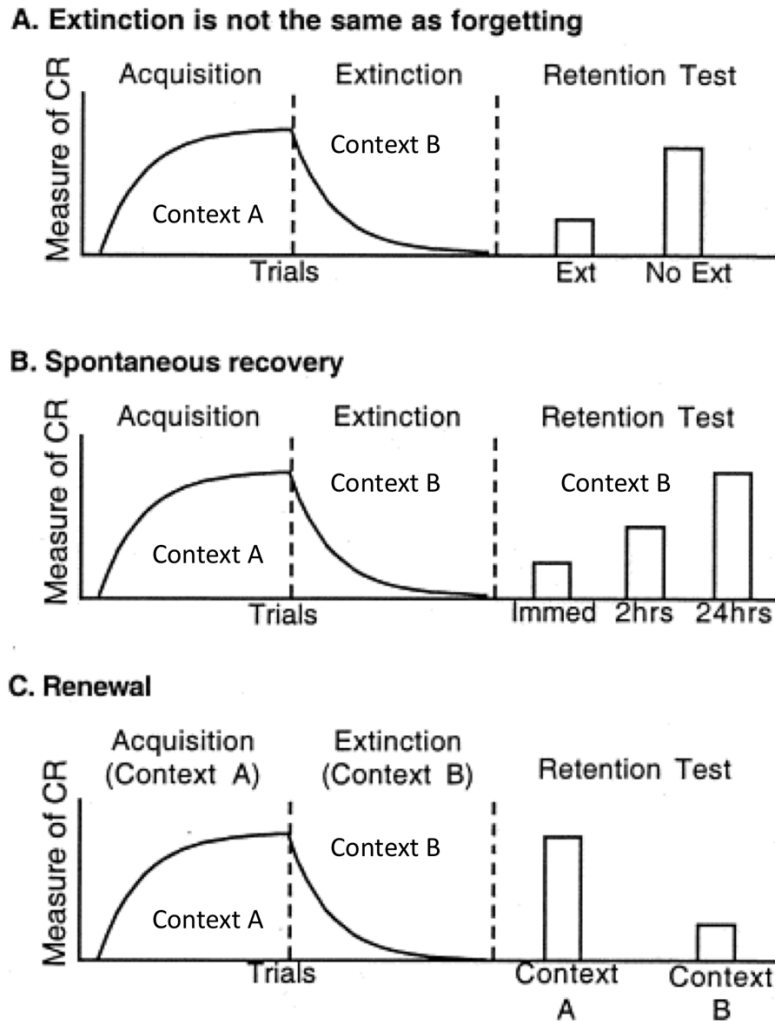


## C. Renewal



Figure 1.3 **Extinction, Spontaneous Recovery, and Fear Renewal** A) Extinction occurs with repeated and unreinforced presentations of the CS in a novel context. B) Fear behavior spontaneously recovers occurs as a function of time from extinction learning. C) Fear behaviors are renewed upon placement in the fear conditioning context. Adapted from [97].

*5.5 Neurotransmitters*

Fear conditioning and fear extinction are mediated through a balance of excitatory and inhibitory neurotransmitters. Glutamate, an excitatory neurotransmitter, initiates fear memory formation and activation of fear behavior and physiologic response circuits.[98]  Glutamate is released from pre-synaptic neurons and binds to ionotropic (AMPA, NMDA, and kainate) and metabotropic receptors on post-synaptic neurons [99]. To initiate long-term potentiation (storage

14

of long-term memory), glutamate binds to AMPA receptors, which opens the sodium channel and allows for the influx of sodium ions. The influx of sodium ions results in depolarization of the post-synaptic membrane. Depolarization releases the magnesium ions that blocks neighboring NMDA receptors.[99] Simultaneous glutamate binding to the NMDA receptor along with corresponding depolarization and magnesium ion release, allows for the influx of calcium ions into the post-synaptic cell.[100] Calcium, a second messenger, then triggers a series of protein kinases that activate proteins and signals the production of new proteins called neurotrophins. One of these neurotrophins is brain-derived neurotrophic factor (BDNF). BDNF increases synaptic plasticity, improves communication between neurons, and enhances memory.[63]

On the other hand, γ-aminobutyric acid (GABA) is a neurotransmitter that maintains an inhibitory tone across fear behavior circuitry. GABA binds to presynaptic GABA receptors with a primary function to suppresses excitatory neurons [101, 102]. GABA binds to $GABA_A$ receptors, which leads to a conformational shape change to open the cation channel [103]. Once open, the channel allows for the influx of chloride cations, which hyperpolarizes the neuronal membrane. Hyperpolarization thereby prevents the depolarization of the membrane and suppresses the electrical threshold activation and maintains inhibitory tone.

Pharmacologic agents that target glutamatergic or GABA neurotransmitter systems may dysregulate fear memory circuitry and impact PTSD [104]. Ketamine, a non-competitive antagonist at the glutamatergic NMDA receptor, is of interest because it is often administered in the immediate period following traumatic injury when fear memories are undergoing consolidation and vulnerable to pharmacologic influence.

## 6. Ketamine

*6.1 Background*

Ketamine is a dissociative anesthetic and potent analgesic used in both humans and animals. Dr. Calvin Stevens, Ph.D., Professor of Organic Chemistry at Wayne State University, first synthesized compound CI-581 (later named ketamine) in 1962 as a less potent and safer derivative of phencyclidine (PCP) that produced short acting anesthesia in animals [105]. CI-581 first underwent human trials in 1964 under the direction of Dr. Edward Domino, M.D. and Dr. Guenter Corssen, M.D. at the University of Michigan [106]. Study participants reported side effects such as "spaced out," "dreaming," and "disconnected." Dr. Domino relayed these reports to his wife, Toni, who then coined the term "dissociative anesthetic" as more apt description of ketamine [105]. The U.S. Food and Drug Administration (FDA) approved ketamine for clinical use in 1970 [107] and ketamine  is now recognized as an essential anesthetic medication by the World Health Organization due to its cost-effectiveness, wide safety margin, and clinical efficacy [108].

*6.2 Pharmacology*

Ketamine (2-chlorophenyl-2-methylamino-cyclohexanone) is a non-competitive antagonist at the NMDA receptor and exists as two (S+ and R-) stereoisomers. Ketamine is prepared and distributed as a racemic mixture of the two isomers (R,S) within the U.S., while the more potent S+ isomer is commercially available outside the U.S. marketplace. Clinicians typically administer ketamine via the intravenous (i.v.) and intramuscular (i.m.) routes to humans [109] while pre-clinical researchers mostly administer ketamine via intraperitoneal (i.p.) and subcutaneous (s.c.) injections to rodents [110]. The high lipid solubility of ketamine supports

rapid distribution from the plasma across the blood brain barrier and into the central nervous system (CNS) where it exerts anesthetic and analgesic effects [105, 111]. Ketamine binds to the PCP binding site within open NMDA channels inside the CNS to block the influx of calcium ions and suppress neuronal activity. Following its rapid absorption and distribution, ketamine undergoes rapid elimination with half times of 2-3 hours in humans [105] and 1.3 hours in 10-week-old rats [112]. Extensive first pass hepatic metabolism utilizes CYP450 cytochromes to hydroxylate ketamine to norketamine, hydroxyketamine, and hydroxynorketamine active metabolites, which are renally excreted as water soluble compounds [105, 113].

*6.3 Side Effects*

Although ketamine preferentially binds to NMDA receptors, it also has weak affinities for mu/kappa opioid, $D_2$ dopamine, 5-$HT_{2A}$ serotonin, sigma, and acetylcholine receptors [114], which contribute toward a host of non-specific systemic effects. Emergence from ketamine anesthesia frequently induces transient psychosis such as hallucinations, delusions, dissociation, and delirium. These psychomimetic effects closely resemble the positive, negative, and cognitive symptoms associated with schizophrenia, which has led investigators to utilize ketamine to model the disease in both human and animal subjects [9, 114]. Beyond psychosis, ketamine is known to induce sympathomimetic effects such as increased heart rate, blood pressure, and bronchodilation [109]. Interestingly, low-dose ketamine infusions suppress cortisol while higher-dose infusions increase cortisol levels similar to an acute stress response suggesting biphasic effects on the HPA axis [9].

*6.4 Off-label use*

Ketamine is gaining interest as an off-label treatment for MDD [115], chronic pain [116], and PTSD [117] that are resistant to traditional treatment regimens. Investigators also report ketamine treatments to reduce suicidality in emergency department settings [118]. Although the

underlying mechanism regarding these ketamine effects is under investigation, many researchers

have associated the ketamine effects with BDNF [119]. BDNF is a neurotrophin associated with

synaptic health, communication between neurons, and learning and memory. Patients diagnosed

with MDD have reduced levels of BDNF [120], while the relationship between PTSD and BDNF

levels are less clear [121, 122]. There is evidence to suggest that low-dose ketamine treatments

increase BDNF levels [119], which provide a transient reduction in depressive symptoms. A

proposed mechanism is that ketamine antagonizes presynaptic NMDA receptors, which

disinhibit GABAergic control over glutamate expressing neurons [119]. GABAergic

disinhibition induces a paradoxical increase in presynaptic glutamate release, which bind to

postsynaptic alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors.

AMPA activation triggers a complex series of second messenger systems that induce the *de novo*

production of BDNF [119], which strengthens connectivity between neurons. Although off-label

ketamine treatments appear promising, there are significant concerns regarding placebo-

expectation effects, high relapse rates, increased tolerance, unknown effects of longitudinal

repeated infusions, and the high abuse potential of ketamine [123-126].

*6.5 Battlefield Administration and Concerns*

Ketamine has gained popularity within forward-deployed military environments as a

seemingly ideal trauma anesthetic. Ketamine induces rapid dissociative anesthesia and potent

analgesia, while maintaining hemodynamic stability and protection of laryngeal reflexes without

respiratory depression [127]. Medical personnel first administered ketamine to the combat

wounded in the Vietnam War and its use has continued into the more recent Iraq and

Afghanistan Wars [4, 5, 105]. The committee on Tactical Combat Casualty Care, an independent

working group within the U.S. DoD Health Board, formally approved combat medics and

paramedics to administer ketamine as an alternative to morphine for battlefield analgesia, due to

its rapid onset and increased safety profile [3]. In addition to battlefield administration, clinicians administer ketamine to the combat wounded as part of forward-deployed surgical resuscitation and en route care during transportation to higher echelons of medical care [7]. Despite its seemingly ideal trauma anesthetic characteristics, ketamine can produce transient psychosis effects such as hallucination, delusions, delirium, dissociation, and agitation [9, 128, 129]. The combination of psychomimetic effects, glutamatergic dysregulation, and receptor non-specificity, raise concerns regarding the impact of post-trauma ketamine administration on fear memory processing and stressor related disorders.

*6.6 Human studies*

There are a limited number of human investigations reporting the effects of post-trauma ketamine on stress related disorders. McGhee et al. conducted two retrospective reviews of U.S. service members who sustained combat related burn injuries and received intraoperative ketamine administration at the U.S. Army Institute of Surgical Research burn unit (San Antonio, TX) between the years 2002-2007 and 2004-2011. The initial review reported an inverse correlation between PTSD diagnosis and intraoperative i.v. ketamine [130]; however, a follow up review with larger sample sizes and greater statistical power found no relationship between PTSD and ketamine [131]. Although these studies are frequently cited in support of peri-trauma ketamine administration, they have significant methodological limitations that should temper the positive findings. The authors did not report the co-morbidity of other mental health disorders or injuries frequently associated with PTSD, such as mild traumatic brain injury (mTBI) or depression. Additionally, the investigators did not control for other psychoactive medications administered in addition to ketamine, which limit the interpretation of ketamine specific effects. The authors utilized a simple binary ketamine inclusion criteria (yes/no) and did not record the number of ketamine administrations, the use of infusion vs. bolus dosing, or total ketamine

dosages. The most significant limitation, however, is the unreported time delay between the initial combat injury and the intraoperative ketamine administration. If clinicians significantly delayed ketamine administration after the initial injury, ketamine would likely have little to no impact on fear memory processing. Moreover, the authors do not report ketamine administration on the battlefield or throughout escalating echelons of care en route to the stateside facility, which could also impact traumatic fear memory and potential PTSD development.

In contrast, Schonenburg et al. conducted two investigations that addressed the effect of i.v. ketamine administered immediately after injury on subsequent PTSD symptomology. First, his team surveyed trauma victims up to one year after receiving either ketamine or opioid analgesia following traumatic injury [132]. Ketamine treated patients reported greater PTSD symptoms such as dissociation, re-experiencing, and avoidance compared to those that received opioids. A follow up study prospectively measured acute stress disorder (ASD) and peri-traumatic dissociation symptoms three days after receiving ketamine, opioid, or non-opioid analgesic treatment following mild to moderate traumatic injury [133]. Once again, patients reported greater dissociation, re-experiencing, avoidance, and hyperarousal symptoms compared to the opioid and non-opioid treatment groups. Interestingly, these results are in agreement with Winter & Irle who reported a significant association between ketamine/benzodiazepine treatment and PTSD diagnosis among burn trauma patients [134]. These investigations also have significant limitations. The studies consisted of non-randomized small samples that did not report longitudinal PTSD symptoms, comorbid mental health disorders, or total opioid and ketamine treatment doses. Despite these limitations, the results reported by Schonenburg et al. and Winter & Irle raise concerns regarding an adverse psychological impact of immediate post-trauma ketamine administration on PTSD symptoms.

*6.7 Animal Studies*

Pre-clinical investigations exploring the effect of post-fear conditioning ketamine administration on PTSD-like behaviors are limited and mixed. Recent rodent fear conditioning studies have reported either improved [135], no change [136], or worsened [137-139] PTSD-like behaviors after ketamine injections. Zhang et al. administered daily sub-anesthetic ketamine injections (0.625, 1.25, or 2.5 mg/kg i.p.) x 18 days, beginning 1-day after a stress model in rats and inescapable footshock (IFS) in mice and found that ketamine improved anxiety-like behaviors, reduced contextual freezing, and increased hippocampal BDNF levels compared to saline controls [135]. In contrast, Groeber et al. administered hourly anesthetic (high-dose) ketamine injections (100/50/50/50 mg/kg i.p.) x 4 hours, beginning immediately after fear conditioning in rats, and reported no change in fear acquisition or cued fear extinction measured over 6 fear extinction trials [136]. Juven-Wetzler et al. administered daily sub-anesthetic ketamine injections (0.5, 5, or 15 mg/kg i.p.) x 3 days beginning 1h after exposure to a predator scent stress (PSS) paradigm and reported increased freezing 31 days later [137]. Morena et al. administered a single injection of ketamine (125 mg/kg i.p) immediately after inhibitory avoidance and IFS paradigms and found that ketamine increased avoidance latency and impaired social interaction among rats tested 48h later [138]. Lastly, Saur et al. administered a single ketamine injection (10 mg/kg i.p.) 6 days after IFS and reported enhanced freezing to a cue reminder 24h later compared to saline controls [139] .

There is an equally limited number of pre-clinical investigations exploring the effects of post-fear conditioning ketamine administration on fear extinction and fear extinction reversal. Similar to PTSD-like behavior studies, ketamine effects on fear extinction are mixed showing either improvement [140] or no change [141, 142], while NMDA antagonists similar to ketamine show detrimental effects [143, 144]. Girgenti et al. administered a single ketamine injection (10

mg/kg, i.p.) to rats 1-day after fear conditioning and reported enhanced fear extinction on the second of three extinction days, and reduced freezing to a cue renewal [140]. In contrast, Yang et al. administered long-term daily ketamine (0.625, 1.25, or 2.5 mg/kg i.p) x 22 days beginning 1h after fear conditioning in mice and reported no difference in fear extinction; however, long-term ketamine reduced spontaneous recovery and cued fear renewal suggesting ketamine prevented the fear extinction reversal [142]. Lastly, McGowan et al. administered a single ketamine injection (30 mg/kg i.p.) either 1h before, 1h after, or 1-week after fear conditioning in mice and observed no change in fear extinction [141]. Interestingly, mice that received ketamine (30 mg/kg i.p.) 1-week before fear conditioning demonstrated significantly reduced freezing during subsequent fear extinction testing suggesting a prophylactic effect of ketamine.

*6.8 Limitations and Gaps*

Conflicting results regarding the ketamine effect on PTSD-like behaviors are not surprising considering the variations in ketamine dosages, administration times, rodent PTSD models, and rodent species. However, a major *limitation* that is common throughout rodent fear conditioning literature is the administration of ketamine via the i.p. injection delivery route. An i.p. injection involves the placement of a needle into the peritoneal space around the abdominal organs. Injecting into the i.p. space is a blind technique with no visualization of needle placement that results in a 10-20% failure rate as inadvertent puncture can occur into fat, urinary bladder, muscle, or abdominal organs [145]. Additionally, repeated needle insertion into the abdomen of a physically restrained rodent on a daily or hourly schedule is distressing, which could impact psychological measures. Lastly, the estimated half-life of an i.p. ketamine injection is around 30 minutes [146], which inadequately covers the length of the estimated 2-4h memory consolidation period in rats [62, 65, 147].

A more clinically relevant administration route is via continuous i.v. infusion. An i.v. ketamine infusion not only translates to the most widely used administration route in humans, but allows researchers to ensure drug delivery and maintain a steady state drug level in the blood and brain to maximize impact on neurobiological mechanisms associated with memory. The lack of pre-clinical literature that explores the effects of sub-anesthetic i.v. ketamine infusions on rodent fear memory processes is a significant *gap* that researchers must explore.

## 7. Purpose and Significance

Combat wounded service members receive ketamine on the battlefield and during initial trauma resuscitation following traumatic injury, but the psychological impacts of ketamine on trauma and stress related disorders are under explored. Therefore, the guiding *purpose* of our investigation is to examine the effect of sub-anesthetic i.v. ketamine infusions on fear memory processes utilizing a rodent fear conditioning model. The *significance* of this study is that understanding the effects of post-trauma ketamine on fear memory and PTSD-like behaviors in rodents will provide insights that will guide future clinical investigations and the treatment policy of the traumatically wounded.

## 8. Specific Aims and Hypotheses

1.  Our initial step is to determine the effective sub-anesthetic i.v. ketamine doses (bolus and infusion) in rats as there is an absence of sub-anesthetic dosing information within the pre-clinical literature. Ketamine infusions are used clinically to provide analgesia; therefore, our aim was to determine sub-anesthetic dosages that could provide analgesia while minimizing dissociative effects. We administered ketamine as a bolus and continuous infusion and measured effects on dissociative stereotypy, locomotor activity, acoustic startle response, pre-pulse inhibition, and nociception in male Sprague-Dawley rats. A secondary aim is to

measure the plasma concentrations of ketamine and norketamine, an active metabolite, following i.v. bolus and a continuous infusion.

*Hypothesis:* The administration of sub-anesthetic i.v. ketamine will dose-dependently increase dissociative stereotypy, locomotor activity, and antinociception, while impairing acoustic startle and pre-pulse inhibition measures.

2.  With knowledge of sub-anesthetic i.v. ketamine dosing in rats, our next intermediate step is to explore i.v. ketamine effects on biomarkers associated with stress and memory. Therefore, our second specific aim is to characterize the dose-dependent effects of sub-anesthetic i.v. ketamine infusions administered post-fear conditioning on CORT and BDNF in the plasma of male Sprague-Dawley rats.

    *Hypothesis*: Intravenous ketamine infusions will dose-dependently increase plasma CORT and BDNF levels in rats.

3.  Our final step is to explore the effects of i.v. ketamine infusions on rodent fear memory. Therefore, our third specific aim is to characterize the dose-dependent effects of sub-anesthetic i.v. ketamine infusions administered either immediately or delayed after fear conditioning on fear memory retrieval, fear memory extinction, and fear memory renewal in male Sprague-Dawley rats.

    *Hypothesis*: Based upon previous literature and a small pilot study, we hypothesize that sub-anesthetic i.v. ketamine infusions will dose-dependently enhance fear memory retrieval, impair fear extinction, and enhance fear renewal to context and cue.

## 9. Manuscript Outline

We prepared three manuscripts for the dissemination of our findings. The first manuscript (Chapter 2) is published and describes the dose-responses of sub-anesthetic i.v. ketamine on dissociative stereotypy, locomotor activity, nociception, and sensorimotor gating in male

Sprague-Dawley rats. This manuscript also reports ketamine and norketamine plasma drug levels after various ketamine dosages. The second manuscript (Chapter 3) is currently under peer-review and describes the dose-dependent effects of sub-anesthetic i.v. ketamine infusions administered after rodent fear conditioning on plasma CORT and BDNF levels, which are biomarkers associated with stress and memory, respectively. The third manuscript (Chapter 4) is prepared for submission and examines the dose-dependent effects of immediate and delayed sub-anesthetic i.v. ketamine infusions administered after rodent fear conditioning on fear memory retrieval, fear memory extinction, and fear memory renewal.

# CHAPTER 2

Dose-response characteristics of intravenous ketamine on dissociative stereotypy, locomotion, sensorimotor gating, and nociception in male Sprague-Dawley rats

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**Abstract**

Clinicians administer sub-anesthetic intravenous (IV) ketamine infusions for treatment of refractory depression, chronic pain, and post-traumatic stress disorder in humans. However, ketamine is administered via the subcutaneous (SC) or intraperitoneal (IP) routes to rodents in most pre-clinical research, which may limit translational application. The present study characterized the dose-response of a sub-anesthetic IV ketamine bolus (2 and 5 mg/kg) and 1-hr infusion (5, 10, and 20 mg/kg/h) on dissociative stereotypy, locomotion, sensorimotor gating, and thermal nociception in male Sprague-Dawley rats. The secondary aim was to measure ketamine and norketamine plasma concentrations following IV ketamine bolus at 1, 20, and 50 min and at the conclusion of the 1-h infusion using liquid chromatography/mass spectrometry. The results showed that ketamine bolus and infusions produced dose-dependent dissociative stereotypy. Bolus (2 and 5 mg/kg) and 20 mg/kg/h infusion increased locomotor activity while 5 mg/kg/h infusion decreased locomotor activity. Both 10 and 20 mg/kg/h infusions reduced the acoustic startle reflex, while 5 mg/kg bolus and 20 mg/kg/h infusion impaired pre-pulse inhibition. Ketamine 5 mg/kg bolus and the 10 and 20 mg/kg/h infusions induced significant and prolonged antinociception to the hotplate test. Plasma concentrations of ketamine decreased quickly after bolus while norketamine levels increased from 1 to 20 min and plateaued from 20 to 50 min. The peak ketamine plasma concentrations [ng/mL] were similar between 5 mg/kg bolus [4100] vs. 20 mg/kg/h infusion [3900], and 2 mg/kg bolus [1700] vs. 10 mg/kg/h infusion [1500]. These results support the findings from previous ketamine injection studies and further validate the feasibility of administering sub-anesthetic doses of IV ketamine infusion to rats for neuropharmacological studies.

*Keywords:* ketamine, rat, intravenous, locomotion, sensorimotor gating, behaviors

## 1. Introduction

Ketamine, a non-competitive antagonist at the glutamatergic *N*-methyl-*D*-aspartate (NMDA) receptor, is a potent analgesic and dissociative anesthetic with many applications in both rodent behavioral and human psychiatric research. Pre-clinical researchers utilize ketamine in various rodent models of psychosis, depression, addiction, and pain while clinicians administer ketamine for treatment of refractory depression, post-traumatic stress disorder (PTSD), and chronic pain. Ketamine is typically administered via subcutaneous (SC) or intraperitoneal (IP) injection to rodents, but these delivery routes may not translate well to human studies and treatments that primarily use continuous intravenous (IV) ketamine infusions. Previous research has focused on evaluating the effects of IP and SC ketamine on rodent behaviors, but little is known regarding the dose-response characteristics of sub-anesthetic doses of ketamine administered by the IV route.

Dose-dependent changes in locomotion and stereotypic dissociative-like behaviors in rodents are observed following sub-anesthetic ketamine injections. At higher doses, ketamine induces dose-dependent hyper-locomotion [148-150] through increased dopamine turnover in limbic-striatal regions [151-153]. Interestingly, several studies have observed reduced locomotion following low-dose ketamine injections [154-156]. Phencyclidine (PCP) and ketamine not only impact locomotion, but also produce other characteristic changes in rodent behavior. Commonly described stereotypic behaviors include circling, side to side head motion, reduced rearing, ataxia, and reduced fine motor movements such as grooming [148, 150, 155, 157]. Similar to hyper-locomotion, dissociative stereotypy is mediated through dopaminergic pathways in striatal regions [158-161].

In addition to stereotypy and locomotion changes, ketamine impacts sensorimotor gating in both humans and rodents [162, 163]. Pre-pulse inhibition (PPI), a measure of sensorimotor

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gating and an indirect measure of information processing, occurs when a weak stimulus precedes a startle stimulus and reduces the acoustic startle reflex (ASR). Ketamine-induced disruption of PPI is well-described [163] and is used to model schizophrenia-like behaviors in rodent psychosis models; however, the effects of ketamine on the ASR are inconsistent. Most investigators show ketamine to have either no effect or a non-significant reduction in ASR [146, 149, 164], while others report an increase in startle response [165].

Ketamine, a potent analgesic in humans [166], produces dose-dependent antinociception in rodents at high doses [167, 168]. Ketamine mediates antinociception via central and peripheral mechanisms primarily through NMDA antagonism and partially through opioid receptor agonism [169, 170]. Although researchers have described dose-dependent increases in hotplate and tail-flick latencies in rodents [167, 171], there are limited reports that describe the duration of antinociception following ketamine administration. The impact of ketamine on nociception over time provides useful information to researchers regarding dose-response characteristics and the duration required to determine optimum antinociceptive doses in rodents.

Overall, there is an abundance of literature characterizing rodent behaviors, information processing, and antinociception following ketamine injections, but there is little information describing the dose-response characteristics of sub-anesthetic IV ketamine and corresponding drug plasma concentrations in rodents. Therefore, our primary aim was to characterize the effects of sub-anesthetic IV ketamine administered as a bolus and continuous infusion on dissociative stereotypy, locomotor activity, ASR, PPI, and nociception in male Sprague-Dawley rats. Our secondary aim was to measure plasma concentrations of ketamine and norketamine, an active metabolite, following IV bolus at 1, 20, and 50 min and at the completion of a 1-h infusion to provide a comparison to previous rodent ketamine studies that used SC or IP injections. Based upon the previous literature, we hypothesized that administration of sub-anesthetic IV ketamine

may dose-dependently increase dissociative stereotypy, locomotor activity, and antinociception while impairing PPI in rats.

## 2. Materials and Methods

*2.1 Animals*

Adult male Sprague-Dawley rats (Taconic Biosciences Inc., Hudson, NY) weighing 300 - 350 g were housed individually in clear Plexiglas shoebox cages (reversed 12-h light/dark cycle; testing during the dark cycle) in a climate-controlled environment with food and water *ad libitum*. Animals were handled daily to limit stress-responses during behavioral testing. All procedures were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and was approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of the Health Sciences.

*2.2 Catheter Surgery*

Rats were anesthetized with ketamine/xylazine (80 and 10 mg/kg, IP) and a jugular vein catheter was cannulated as previously described [172]. Briefly, the right jugular vein was isolated through a neck incision and a 3Fr polyurethane catheter (Instech, Plymouth Meeting, PA) was advanced into the vein. The remaining catheter exited via a back incision. The externalized catheter was made with blunt tip 22-gauge stainless steel cemented into place with bell-shaped dental cement. Catheters were flushed daily with a saline solution (0.2 mL) containing gentamycin (1 mg/ml) and heparin (10 units/ml). Animals were given a 7-day recovery period prior to testing.

*2.3 Bolus Experiment*

**2.3.1 Drug and Dosing.** Racemic (+/-) ketamine hydrochloride (100 mg/ml) (Mylan Institutional LLC, Rockford, IL) was diluted in 0.9% sterile saline daily prior to bolus and infusion experiments. A counter-balanced design of ketamine doses (saline, 2 mg/kg, and 5

mg/kg, IV) was used ($n$ = 8 per dose). Animals were tested on Days 1 and 5 separated by a 4-day drug clearance. Control group animals received IV saline while experimental groups received ketamine doses 2 or 5 mg/kg IV immediately prior to behavioral testing. All doses were delivered in a 1 mL/kg volume.

**2.3.2 Dissociative stereotypy.** Following ketamine bolus, individual animals were placed in a clear Plexiglas shoebox cage (42.5 x 20.5 x 20 cm). Five stereotypic dissociative-like behaviors (circling, side to side head motion (weave), rearing, ataxia, and grooming) were video recorded (Sony DCR-SX44) over 10 min following ketamine administration (Figure 2.1). A study team member blinded to dosing watched the recorded video and tallied the frequency of each behavior and grouped the data into 2-min bins (5 bins total). Dissociative behaviors were chosen based upon previous ketamine and PCP literature [155, 157].

**2.3.3 Locomotion.** Following ketamine bolus, individual animals were placed in a clear Plexiglas shoebox cage (42.5 x 20.5 x 20 cm) marked with 9 equal sized floor grid-squares. Locomotion was video recorded (Sony DCR-SX44) over 15-min (Figure 2.1). A member of the study team blinded to ketamine dosing watched the recordings, counted the total number of square entries, and grouped the data into 3-min bins (5 bins total). A square entry consisted of both front and rear paws entering the square.

**2.3.4 Sensorimotor Gating.** ASR was measured with a Startle Response Acoustic Test System as previously described [173] for the bolus and infusion experiments. The ASR system consisted of 4 weight-sensitive platforms in 4 sound-attenuated chambers (Coulbourn Instruments, Columbus, OH). A ventilation chamber fan provided background noise during a 3-min acclimation period and the testing session. After acclimation, animals were exposed to 6 types of stimulus trials: 100 and 110 dB startle pulse (SP), 100 and 110 dB SP with an 84 dB pre-pulse (PP), 84 dB PP alone, and no stimulus. A piezoelectric accelerometer measured the

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maximum startle amplitude occurring within 200 ms of the onset of the SP. The interval between

PP and SP was 100 ms and each trial type was presented 8 times. Those values were analyzed

using the ROUTE method for outlier detection before calculating the mean using the GraphPad

Prism software (ver 7.0). No animal was removed from the data as an outlier. Trial types were

presented in random order with inter-trial intervals ranging from 15 - 25 s to avoid order effects

of habituation. A desktop computer with ASR System software was used to present stimuli and

record data. PPI was defined as the percentage reduction in mean startle amplitude in the

presence of the PP compared to mean startle amplitude in the absence of PP: 100 - [100 x (mean

startle amplitude PP + SP trials/mean startle amplitude SP alone trials)]. Baseline ASR and PPI

were measured 24-h before the testing. Subsequent ASR and PPI were measured 20 min after

bolus dosing (Figure 2.1).

      **2.3.5 Thermal Nociception.** Nociception was measured using hotplate latency

(Omnitech/Accuscan Electronics Analgesia Monitor) for bolus and infusion experiments. The

metal hotplate (26 x 26 cm) was enclosed on all sides by clear Plexiglas walls, covered with a

removable Plexiglas top, and heated to 51°C. Latencies were measured as the time in seconds

from placement on the heated surface until the animal raised and licked the hind paw. Animals

that did not lick their hind paws during the 60 s period were removed from the surface. Two

cohorts of rats were used to measure nociception. Cohort 1 ($n = 8$) was tested at 20, 30, 40, and

50 min after ketamine bolus while cohort 2 ($n = 8$) was tested at 70 and 90 min. The latencies

from both cohorts were combined to show duration data from 20 - 90 min post bolus (Figure

2.1).

      **2.3.6 Blood Sampling.** A separate group of rats were used to collect blood samples

following ketamine administration. Blood was withdrawn (0.2 ml) to clear the catheter of

medication and prime with fresh blood. Then 0.4 ml of blood was withdrawn and placed into 1.5

ml Eppendorf tubes with 10 µL of ethylene-diamine-tetra-acetic-acid (EDTA) additive to prevent

blood clotting. Blood samples were centrifuged at 4,000 rpm for 20 min at 4°C (Eppendorf 5415

R). Plasma supernatant (100 µL) was collected for liquid chromatography/mass spectrometry

(LCMS). The blood sample did not exceed volume limits recommended by the Animal Care and

Use guidelines of the NIH [174]. Blood samples were collected at baseline and at 1, 20, and 50

min after ketamine bolus ($n$ = 6 per dose) (Figure 2.1).
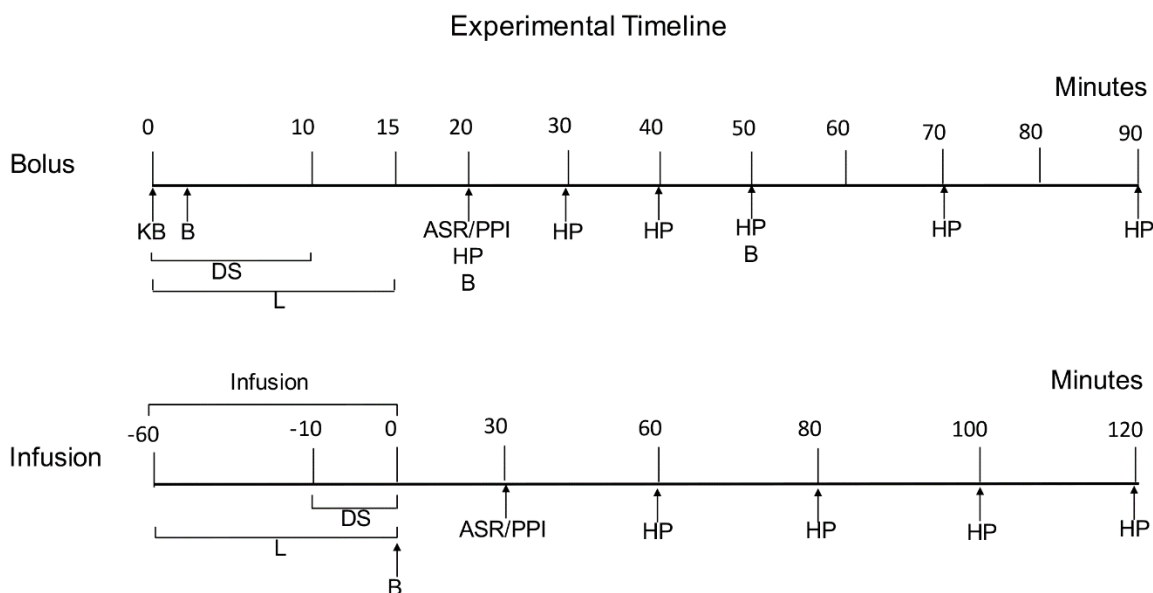


*Figure 2.1.* **Experimental timeline for ketamine bolus and infusion**. KB = ketamine bolus (saline, 2 and 5 mg/kg, IV), B = blood sample, DS = dissociative stereotypy, L = locomotion, ASR/PPI = acoustic startle reflex/pre-pulse inhibition, HP = hotplate latency, Infusion (saline and ketamine 5, 10, and 20 mg/kg/h, IV). Baseline hotplate and ASR/PPI were conducted 24-h prior to first dosing.

*2.4 Infusion Experiment*

**2.4.1 Drug and Dosing.** A separate group of animals from the bolus experiment

underwent ketamine infusions in a counter-balanced design separated by a 4-day drug clearance

between doses ($n$ = 16 per dose). Each infusion trial began with a 5 mg/kg IV ketamine bolus to

ensure plasma loading followed by a 1-h sub-anesthetic IV ketamine infusion at either 5mg/kg/h,

10mg/kg/h, or 20mg/kg/h delivered by syringe pump (Harvard, Holliston, MA). A separate control group not exposed to previous ketamine infusions ($n = 16$) received a 1-h saline infusion. Total 1-h infusion volumes did not exceed 1 mL.

**2.4.2 Apparatus.** Ketamine infusions were administered in an operant conditioning chamber (Med Associates Inc., St. Albans, VT). Chambers were equipped with a 10 ml plastic syringe and Teflon tubing connected to a fluid swivel (Instech, Plymouth Meeting, PA) encased in a metal spring wire. Rats were freely mobile within the operant chamber during the infusion. The room lights were off and a small red chamber light facilitated behavioral observation.

**2.4.3 Dissociative Stereotypy.** A study team member blinded to dosing observed and counted the frequency of dissociative stereotypy during the final 10 min of the 1-h infusion period as described in the bolus experiment (Figure 2.1).

**2.4.4 Locomotion.** Operant chambers were equipped with photo beams to facilitate counting locomotion during the infusion. The total number of beam breaks were recorded throughout the 1-h infusion period and grouped into 10-min bins (6 bins total) (Figure 2.1).

**2.4.5 Sensorimotor Gating.** Baseline ASR and PPI were measured at baseline and 30 min after completion of the 1-h infusion (Figure 2.1).

**2.4.6 Thermal nociception.** Hotplate latency was measured at baseline and 60, 80, 100, and 120 min after completion of the 1-h ketamine infusion (Figure 2.1).

**2.4.7 Blood Sampling.** Blood samples were collected at the conclusion of the 1-h ketamine infusion in the same manner as described in the bolus experiment (Figure 2.1).

*2.5 Liquid Chromatography Mass Spectrometry*

The LCMS protocol was developed based upon previous literature [175]. Plasma samples were precipitated with 5 volumes of cold acetonitrile (500 µL), allowed to stand on ice for 15 min, then spun at 13,000 rpm x 5 min. The supernatant (83%) was removed and dried in a Savant

SpeedVac Concentrator. The pellets after drying were dissolved in a volume of 0.2% formic acid equal to the initial serum volume, then further diluted 50X, also with 0.2% formic acid. LCMS injections (5 μL) were performed on an Agilent 1200 HPLC system feeding a Turbo V ion source on an AB Sciex Q-Trap 4000; a TARGA C18 column (100 X 2.1 mm, 3 um) with a flow rate of 200 μL /min was used to separate ketamine and its metabolites. Multiple Reaction Monitoring transitions for ketamine (238.1 -> 125.0 Da) and norketamine (224.1 -> 125.0 Da) were run for 40 msec, and the identity of each analyte was confirmed with Enhanced Product Ion scans run at 238.1 and 224.1 Da, respectively. Peak areas were measured and converted to concentrations using calibration curves run over a range of 0.5 to 400 ng/ml (concentration in 50X dilutions of serum sample) for both analytes.

*2.6 Statistical Analysis*

All data are presented as means ± standard error of the mean (SEM) and were analyzed using GraphPad Prism (GraphPad Software, version 7.0) with $p$ values < .05 considered significant. Locomotion and nociception data were analyzed with RM two-way analysis of variance (ANOVA), with time and ketamine dose as factors. Total locomotion, dissociative stereotypy, ASR/PPI, and LCMS data were analyzed using one-way ANOVA. Differences were compared with *post-hoc* Tukey's HSD.

## 3. Results

*3.1 Bolus Experiment*

**3.1.1 Dissociative stereotypy.** Dissociative behaviors were observed for 10 min after IV ketamine bolus. There were significant dose-dependent differences in stereotypy (Figure 2.2). Ketamine induced greater circling $F(2,21) = 36.7$ ($p < .001$), reduced rearing $F(2,21) = 34.1$ ($p < .001$), increased head weave $F(2,21) = 18.9$ ($p < .001$), increased ataxia $F(2,21) = 37.1$ ($p < .001$), and reduced grooming $F(2,21) = 21.3$ ($p < .001$) compared to saline. Ketamine 5 mg/kg

bolus induced each dissociative behavior ($p < .001$) while 2 mg/kg reduced rearing and grooming behaviors ($p < .001$) and spared ataxia, circling, and head weave. Circling, ataxia, and head weave resolved within the first 6 minutes after the 5 mg/kg dosing (Figure 2.2); however, reduced rearing and grooming behaviors persisted throughout the 10 min observation period for both ketamine doses.
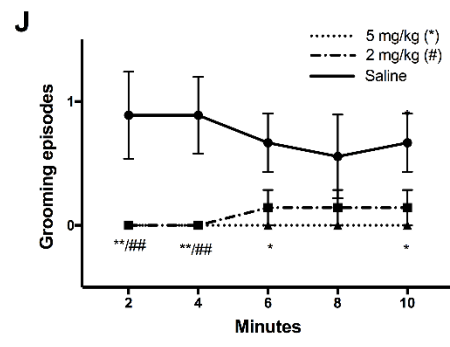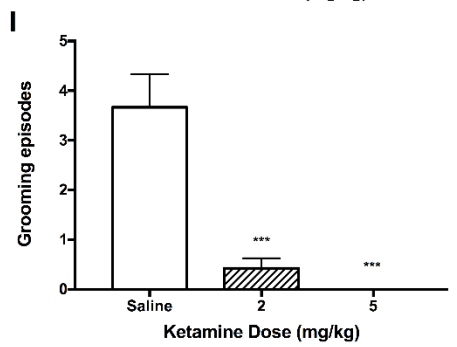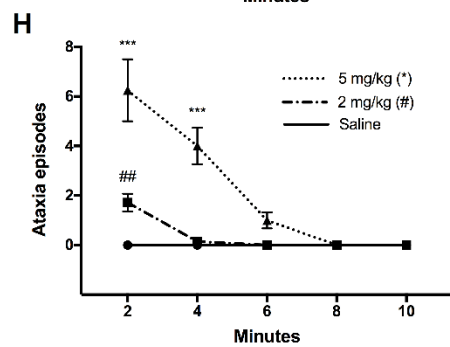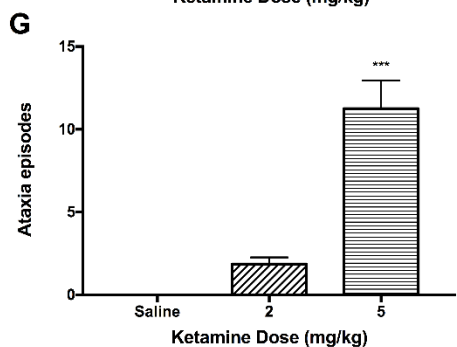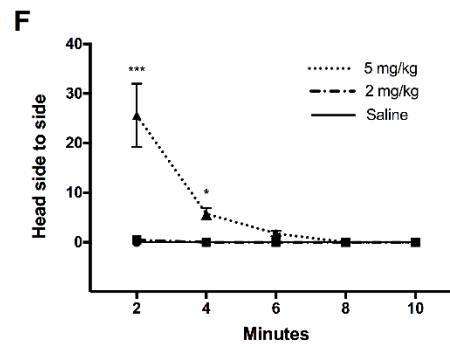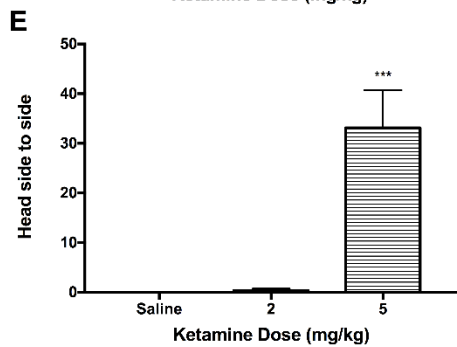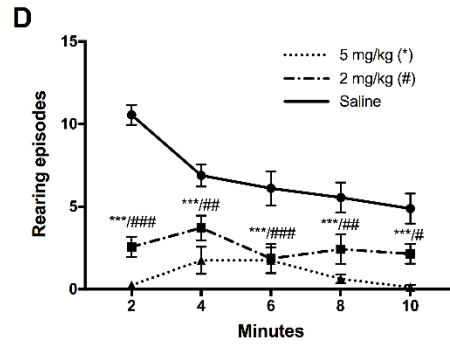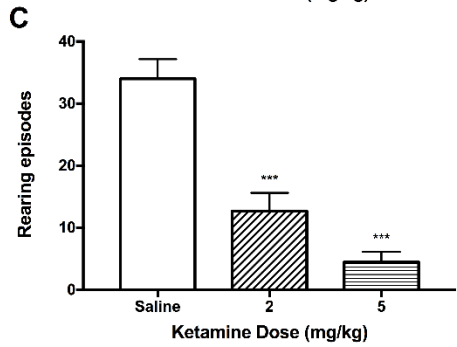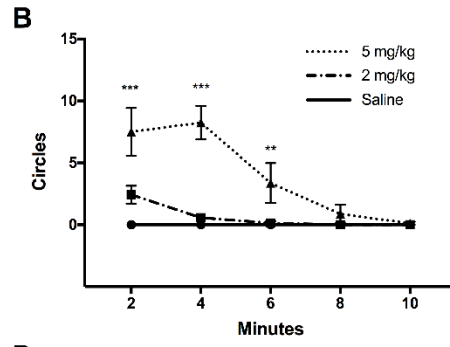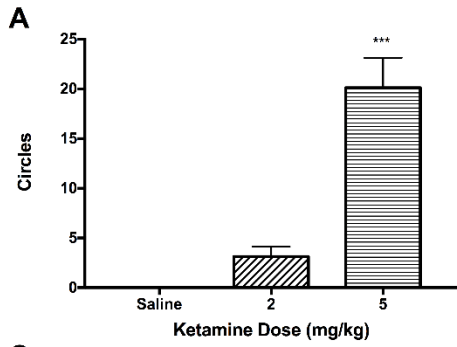
*Figure 2.2.* **Effects of intravenous ketamine bolus on dissociative behaviors**. Dissociative behaviors comprised circling, side to side head motion (weave), reduced rearing, ataxia (loss of balance), and reduced grooming. A-B) Ketamine 5 mg/kg significantly increased total circling with most circles occurring from 0-6 min. C-D) Ketamine 2 and 5 mg/kg dose dependently reduced total rearing over 10 min. E-F) Ketamine 5 mg/kg induced significant total head weave behavior with most occurring from 0-4 min. G-H) Ketamine 5 mg/kg induced more ataxia episodes. Ketamine 2 mg/kg induced ataxia over 2 min while 5 mg/kg induced ataxia over 4 min. I-J) Ketamine dose dependently reduced grooming behavior. Ketamine 2 mg/kg reduced grooming over 4 min while 5 mg/kg reduced grooming throughout 10 min. Data are shown as mean ± SEM. (*/# $p < .05$, **/## $p < .01$, ***/### $p < .001$ vs. saline; $n = 8$ per group).

**3.1.2 Locomotion.** Locomotor activity was measured over 15 min following an IV ketamine bolus. Ketamine significantly increased total locomotor activity, $F(2,21) = 5.23$ ($p < .05$). Post-hoc testing showed that ketamine 5 mg/kg significantly increased total locomotion vs. saline (Figure 2.3a). The effects of ketamine on locomotion over time were also compared (Figure 2.3b). There were significant main effects of dose $F(2,69) = 12.19$ ($p < .001$), time $F(4,276) = 66.04$ ($p < .001$), and time x dose interaction $F(8,276) = 33.55$ ($p < .001$). Post-hoc testing showed that ketamine 2 mg/kg induced immediate hyper-locomotion between 0-3 min while the 5 mg/kg dose induced delayed hyper-locomotion between 4-9 min. Interestingly, ketamine treated rats trended towards hypo-locomotion by 15 min, but did not reach significance. Overall, the stimulatory effects of ketamine were dose dependent as 2 mg/kg caused immediate hyper-locomotion, while 5 mg/kg produced a delayed and extended hyper-locomotion response with greater total activity.
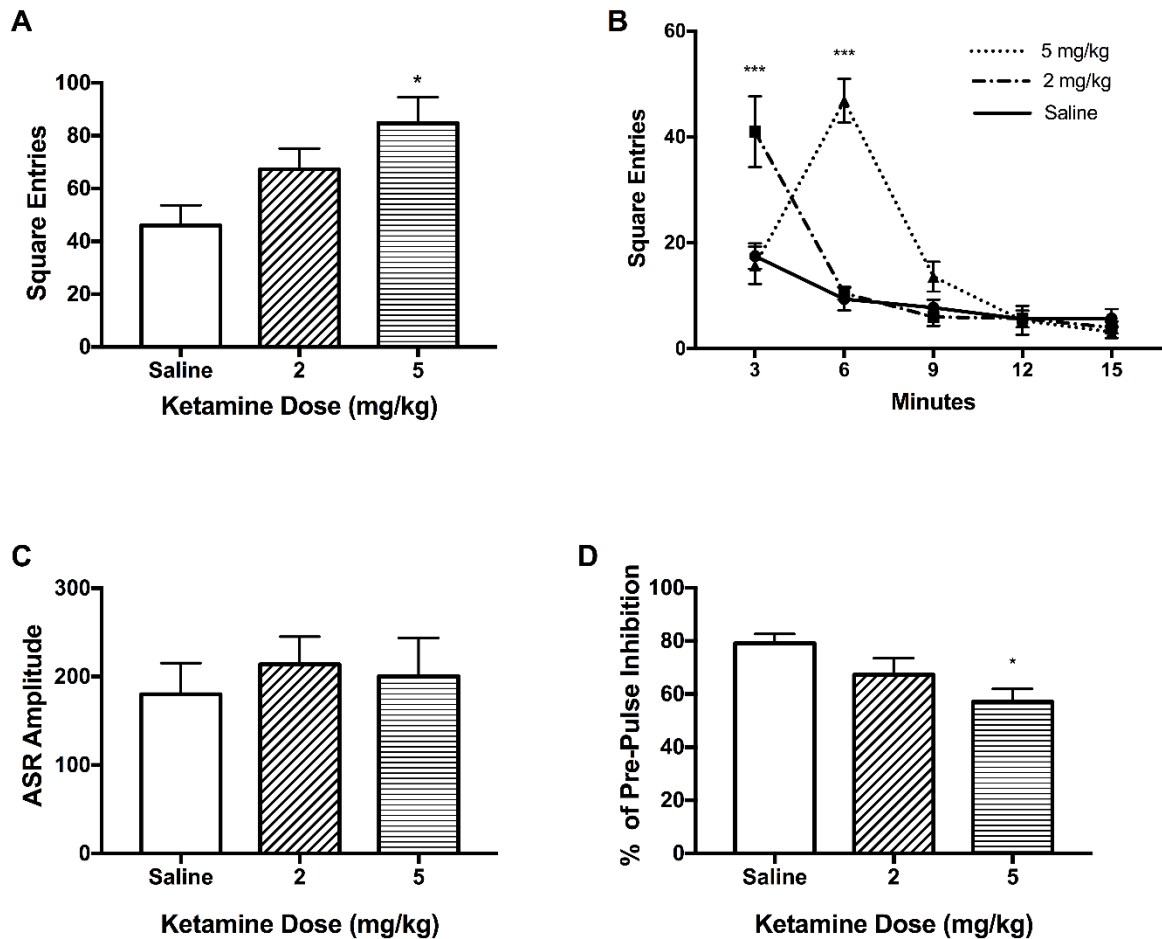
*Figure 2.3.* **Effects of intravenous ketamine bolus on locomotor activity and sensorimotor gating at 100dB.** A) Ketamine 5 mg/kg bolus increased total activity over 15 min. B) Ketamine 2 mg/kg bolus increased activity from 0-3 min while 5 mg/kg produced a delayed response and increased activity from 4-6 min. C) Ketamine did not alter startle response 20 minutes after bolus dosing. D) Ketamine 5 mg/kg impaired PPI while 2 mg/kg had no effect. Data are shown as mean ± SEM. (* $p < .05$; *** $p < .001$ vs. saline; $n = 8$ per group).

**3.1.3 Sensorimotor gating.** The effects of ketamine on ASR and PPI measured 20 min after IV bolus were compared. There were no differences in ASR and PPI results across groups at baseline. Ketamine had no effect on maximum startle amplitude at 100 dB $F(2,21) = 0.212$ ($p = 0.8$) (Figure 2.3c). However, ketamine impaired the PPI of 100 dB, $F(2,21) = 4.89$ ($p = .018$) (Figure 2.3d). Post-hoc testing showed ketamine 5 mg/kg significantly impaired the PPI of 100 dB compared to saline ($p < .05$) while 2 mg/kg showed no effect.

**3.1.4 Thermal nociception.** Hotplate latency was measured from 20 to 90 min after IV ketamine bolus. There were no differences in baseline latencies between groups. There were significant main effects of ketamine dose, $F_{(2,14)} = 14.68$ ($p < .001$) and time, $F_{(5,70)} = 5.265$, ($p < .001$) and no significant dose x time interaction. Post-hoc testing showed ketamine 5 mg/kg produced significant antinociception between 20 - 50 min vs. saline (Figure 2.4a). Although the 2 mg/kg bolus latencies were elevated between 20 - 40 min , the differences did not reach significance. Overall, ketamine 5 mg/kg produced antinociception up to 50 minutes after a bolus injection.
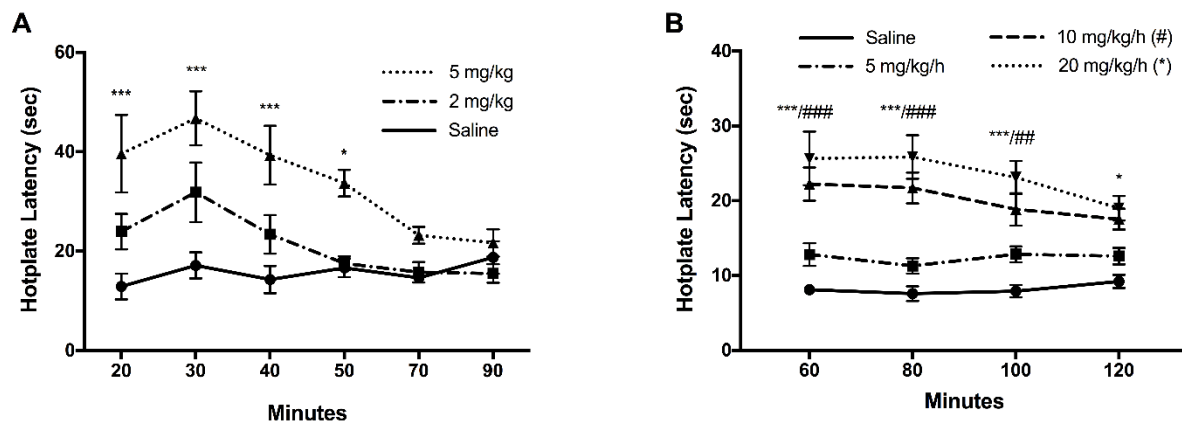


*Figure 2.4.* **Effects of intravenous ketamine bolus and infusion on hotplate latency**. A) Ketamine increased antinociception up to 50 min after a 5 mg/kg bolus injection. B) Ketamine 20 mg/kg/h induced significant antinociception through 120 min after the completion of a 1-h infusion. Ketamine 10 mg/kg/h induced antinociception through 100 min. Data are shown as mean ± SEM. (* $p < .05$, **/## $p < .01$; ***/### $p < .001$ vs. saline; $n = 8$ per bolus group; $n = 16$ per infusion group).

**3.1.5 Plasma concentration.** Ketamine and norketamine plasma concentrations were measured at serial time-points after IV bolus using LCMS (Table 2.1). Ketamine 5 mg/kg produced significantly greater plasma concentrations of ketamine at each time point compared to 2 mg/kg. As expected, there were no differences between norketamine concentrations at 1 min as ketamine had little time to metabolize. Norketamine concentrations increased from 1 to 20 min

and essentially plateaued from 20 to 50 min for both dosages. The 5 mg/kg bolus produced

significantly greater norketamine concentrations at 50 min.

| *Table 2.1.* Plasma concentrations after IV ketamine bolus | | | | |
|---|---|---|---|---|
| | Ketamine | | Norketamine | |
| | 5 mg/kg | 2 mg/kg | 5 mg/kg | 2 mg/kg |
| 1 min | 4100 (690)** | 1700 (170) | 76 (2.5) | 77 (11) |
| 20 min | 490 (27)* | 340 (27) | 290 (28) | 240 (7.6) |
| 50 min | 140 (11)*** | 73 (7.6) | 320 (11)*** | 230 (7.0) |

Table 2.1. **Plasma concentrations of ketamine and norketamine after bolus injections**. Ketamine 5 mg/kg IV

produced greater ketamine levels at each time point compared to 2 mg/kg. Norketamine metabolite levels were

similar at 1 and 20 min between dosages, but 5 mg/kg produced greater norketamine at 50 min. Data are presented

as means (SEM) [ng/ml]. (* $p < .05$, ** $p < .01$, *** $p < .001$; n = 6 per group)

*3.2 Infusion Experiment*

**3.2.1 Dissociative stereotypy.** Dissociative behaviors were observed over the final 10

min of a ketamine infusion. There were significant dose-dependent differences in circling

$F(3,28) = 6.48$ ($p = .002$), rearing episodes $F(3,28) = 14.3$ ($p < .001$), head weave $F(3,28) = 4.87$

($p = 0.008$), ataxia $F(3,28) = 128$ ($p < .001$), and grooming $F(3,28) = 25.3$ ($p < .001$). Rats that

received ketamine at 20 mg/kg/h demonstrated each dissociative behavior (increased circling,

reduced rearing, increased head weave, increased ataxia, and reduced grooming), while rats

dosed at 5 and 10 mg/kg/h demonstrated reduced rearing and grooming (Figure 2.5).
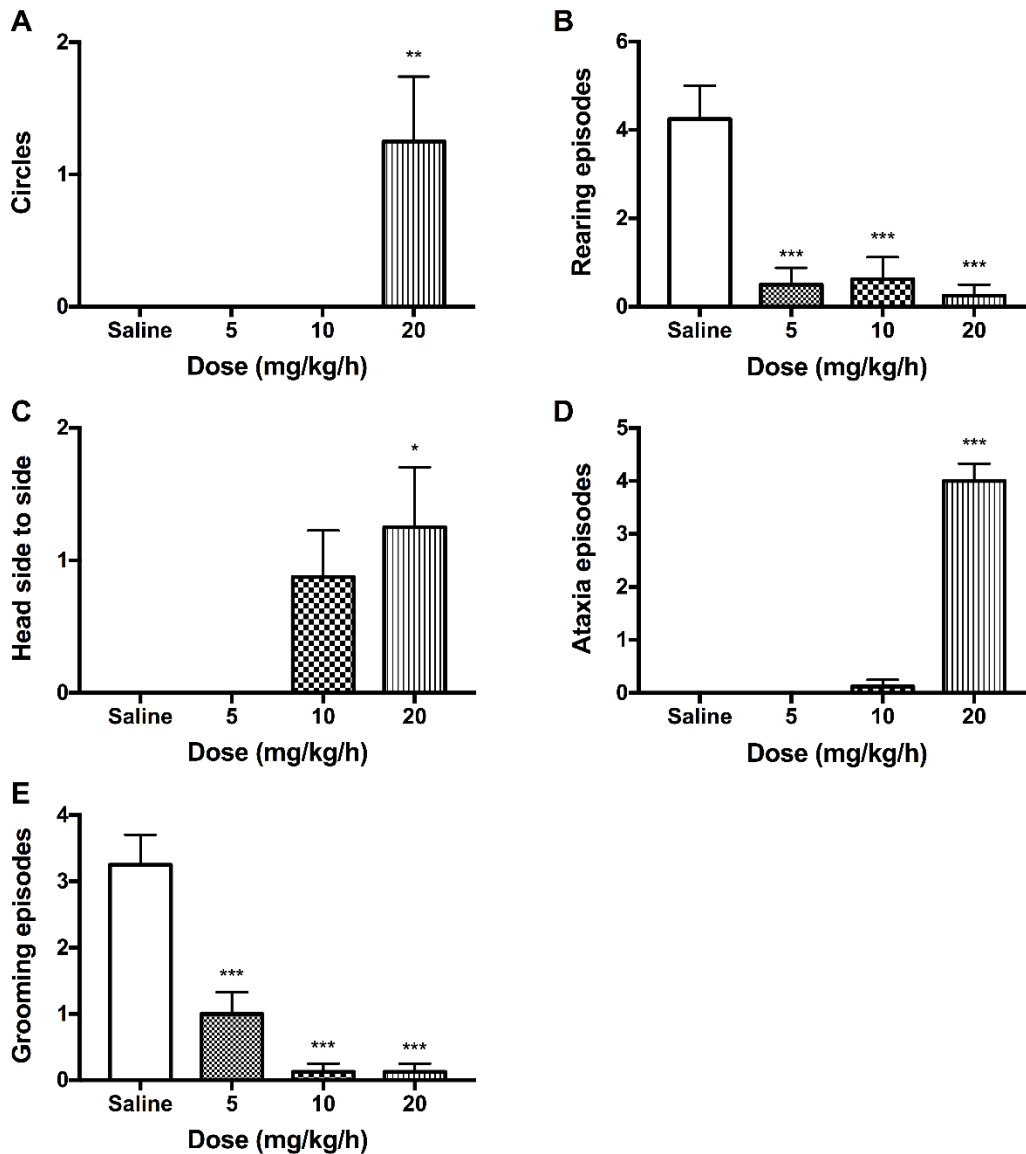
*Figure 2.5.* **Effects of intravenous ketamine infusion on dissociative behaviors**. Dissociative behaviors comprised circling, side to side head motion (weave), reduced rearing, ataxia (loss of balance), and reduced grooming. A) Ketamine 20 mg/kg/h increased circling behavior. B) Ketamine infusions reduced rearing at all doses. C-D) Ketamine 20 mg/kg/h significantly increased head weave and ataxia. E) Ketamine infusions significantly reduced grooming behavior at all doses. Data are shown as mean ± SEM. (* $p < .05$, ** $p < .01$, *** $p < .001$ vs. saline; $n = 8$ per group).

**3.2.2 Locomotion.** Locomotor activity was measured throughout 1-h ketamine infusions. Significant differences were observed across the ketamine groups in total locomotion, $F(3,60) =$

42

19.03 ($p < .001$). Post-hoc testing showed ketamine 20 mg/kg/h significantly increased total activity vs. saline ($p < .001$) while 5 mg/kg/h reduced activity ($p < .05$) (Figure 2.6a). Next, the effects of ketamine on activity over time were measured (Figure 2.6b). There were significant main effects of dose $F(3,62) = 20.65$ ($p < .001$), time $F(5, 310) = 111.1$ ($p < .001$), and a time x dose interaction $F(15,310) = 4.883$ ($p < .001$). All ketamine doses showed increased activity vs. saline vehicle from 0-10 min ($p < .05$), which was most likely due to the ketamine 5mg/kg loading dose. There were no differences in activity between 10-20 min. However, the ketamine 20 mg/kg/h infusion maintained hyper-locomotion vs. saline from 30 to 60 min. Interestingly, ketamine 5 mg/kg/h had an opposite effect and induced hypo-locomotion vs. saline during the same period of 30 to 60 min. Overall, there was a dose-dependent locomotor response to ketamine infusions. The 20 mg/kg/h infusion increased activity while the 5 mg/kg/h infusion reduced locomotion.
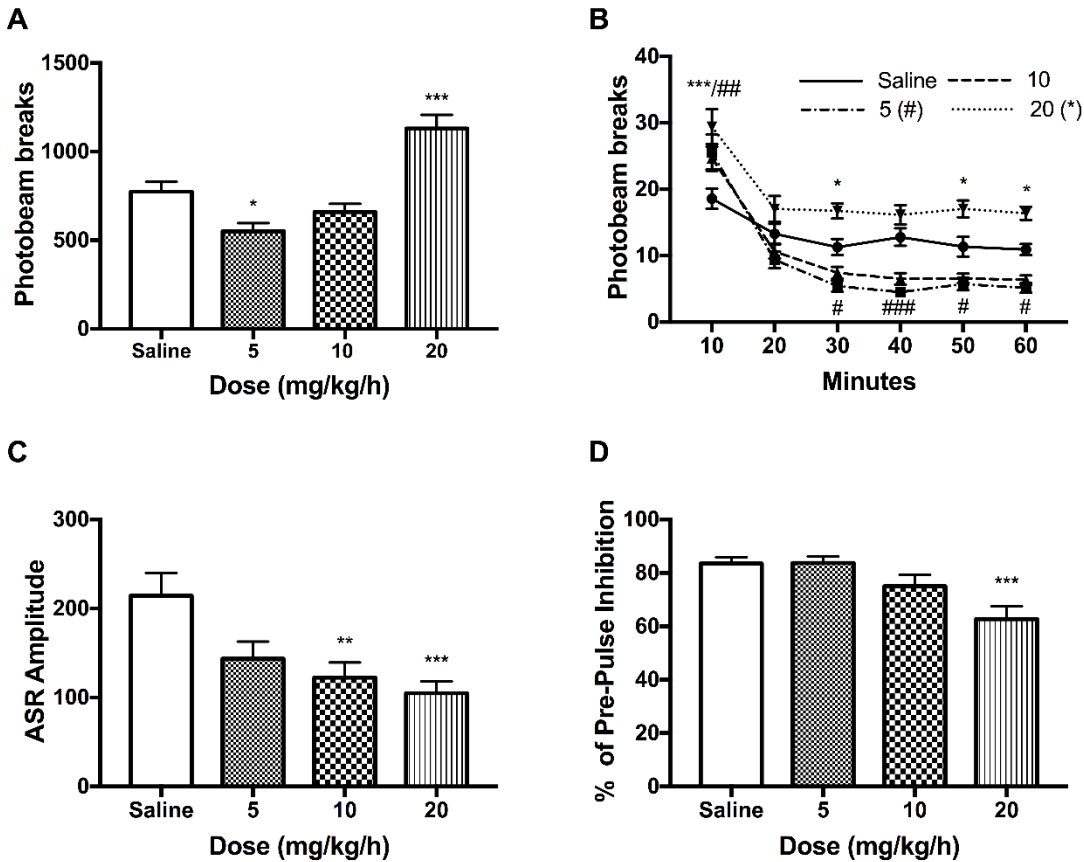
*Figure 2.6.* **Effects of intravenous ketamine infusion on locomotor activity and sensorimotor gating at 100dB**.

A) Ketamine 20 mg/kg/h infusion increased total activity over 60 min while 5 mg/kg/h reduced activity. B) Ketamine 20 mg/kg/h induced hyper-locomotion over the infusion period while 5 mg/kg/h induced hypo-locomotion. C) Ketamine at 10 and 20 mg/kg/h reduced the startle response 30 min after a 1-h infusion. D) Ketamine 20 mg/kg/h impaired PPI while lower dose infusions had no effect. (*/# $p < .05$; **/## $p < .01$; ***/### $p < .001$ vs. saline; $n = 16$ per group).

**3.2.3 Sensorimotor gating.** ASR and PPI were measured 30 min after completion of 1-h ketamine infusions. There were no differences in ASR and PPI results across groups at baseline. Ketamine reduced ASR at 100 dB, $F(3,60) = 6.27$ ($p < .001$). Post-hoc testing revealed that ketamine infusions at 10 and 20 mg/kg/h significantly reduced ASR at 100 dB compared to saline (Figure 2.6c). Ketamine significantly impaired the PPI of 100 dB $F(3,60) = 7.338$ ($p < .001$) (Figure 2.6d). Post-hoc testing showed ketamine 20 mg/kg/h significantly impaired the PPI

at 100 dB compared to saline ($p < .001$). Overall, 10 and 20 mg/kg/h sub-anesthetic ketamine infusions reduced ASR while only 20 mg/kg/h impaired PPI.

**3.2.4 Thermal nociception.** Hotplate latencies were measured from 60 to 120 min after the completion of a 1-h ketamine infusion. There were no differences in baseline latencies between groups. There were significant main effects of ketamine dose, $F(3,52) = 18.68$ ($p < .001$) but non-significant effects for time, $F(3,156) = 1.831$ ($p = .14$) or dose x time interaction, $F(9,156) = 1.369$ ($p = .21$). Post-hoc testing showed that a ketamine 20 mg/kg/h infusion produced significant antinociception up to 120 min after the infusion (Figure 2.4b). The ketamine 10 mg/kg/h infusion produced significant antinociception up to 100 min. Low-dose ketamine at 5 mg/kg/h did not produce antinociception. Ketamine infusions at 20 and 10 mg/kg/h produced significant and extended antinociception compared to the low-dose ketamine infusion.

**3.2.5 Plasma Concentration.** Ketamine and norketamine plasma concentrations were measured at the conclusion of 1-h ketamine infusions (Table 2.2). There were significant differences in ketamine plasma levels between infusion doses, $F(2,15) = 17.21$ ($p < .001$). Ketamine infusion at 20 mg/kg/h produced greater ketamine plasma concentrations compared to both 5 ($p < .001$) and 10 mg/kg/h ($p < .01$) infusions. Although the 10 mg/kg/h infusion resulted in greater ketamine concentration compared to 5 mg/kg/h, the difference was not significant. Similarly, there were significant differences in norketamine levels after the 1-h ketamine infusion, $F(2, 15) = 37.37$ ($p < .001$). Post-hoc testing showed ketamine infusion at 20 mg/kg/h produced greater norketamine than both 5 and 10 mg/kg/h infusions ($p < .001$). We also compared ketamine plasma concentrations between bolus and infusion doses. The 20 mg/kg/h infusion produced a similar ketamine concentration as the 5 mg/kg bolus ($p > .05$). Additionally, the 10 mg/kg/h infusion and the 2 mg/kg bolus resulted in similar ketamine concentrations ($p > .05$). The 5 mg/kg/h infusion produced significantly lower ketamine concentration than the 2

mg/kg bolus ($p < .01$). Overall, there was a dose-dependent effect on ketamine and norketamine plasma concentrations.

| Table 2.2. Plasma concentrations after 1-h IV ketamine infusion | | |
|---|---|---|
| | **Ketamine** | **Norketamine** |
| **5 mg/kg/h** | 880 (140) | 490 (20) |
| **10 mg/kg/h** | 1500 (120) | 740 (43) |
| **20 mg/kg/h** | 3900 (560)** | 1200 (90)*** |

*Table 2.2.* **Plasma concentrations of ketamine and norketamine after a 1-h ketamine infusion.** Ketamine 20 mg/kg/h resulted in greater ketamine and norketamine concentrations than both 5 and 10 mg/kg/h. Data are presented as mean (SEM) [ng/ml]. (** $p < .01$; *** $p < .001$; $n = 6$ per group).

## 4. Discussion

Rodent behaviors following SC and IP ketamine injections are well-described, but clinicians typically administer ketamine treatments to humans using the IV route. Therefore, this investigation sought to provide a missing translational linkage between traditional rodent SC/IP injections and human IV ketamine delivery. To our knowledge, the present investigation is the first to measure dose-response characteristics of rodent behaviors following sub-anesthetic IV ketamine administered as a bolus and continuous infusion. Ketamine administered by injections produce well-described changes in rodent behaviors such as increased locomotion, dissociative stereotypy, impaired PPI, and antinociception. This investigation supported and extended these findings through the characterization of behavioral effects at various IV ketamine dosages, which will facilitate future pre-clinical IV ketamine studies.

High-dose sub-anesthetic IV ketamine delivered as a bolus and infusion induced hyper-locomotion while low-dose ketamine infusions reduced locomotor activity. These results are

similar to previous reports that observed dose-dependent hyper-locomotion following sub-anesthetic ketamine injections [148-150, 176]. In addition to NMDA receptors, ketamine is known to interact with other receptor types that include dopamine receptors [176, 177]. Previous PCP studies suggest that ketamine induces rodent locomotor activity at high-doses through increased dopamine turnover in the nucleus accumbens [151-153, 178]. Interestingly, rats given ketamine at 2 and 5 mg/kg IV boluses trended towards hypo-locomotion after 15 min, and lower-dose 5 and 10 mg/kg/h infusions reduced locomotion compared to saline controls (Figure 2.3 and 6). Previous reports described hypo-locomotion following low-dose sub-anesthetic ketamine injections, but the mechanism is unclear [146, 155, 156, 179]. Clinicians are administering ketamine infusions for treatment refractory PTSD at low-doses that typically do not produce psychotomimetic effects [117]. It is possible that low-dose sub-anesthetic ketamine exerts anxiolytic or calming effects [180] while sparing dopamine induced locomotor effects.

Intravenous ketamine produced dose-dependent changes in dissociative stereotypy. High-dose sub-anesthetic IV ketamine at 5 mg/kg bolus and 20 mg/kg/h infusion induced behaviors such as circling, side to side head movement (head weave), mild ataxia, reduced rearing, and reduced grooming (Figures 2 and 5). These dissociative stereotypy are well-described in previous reports [148, 157, 181] and are mediated by dopaminergic activation in striatal regions [159-161]. Rats that received a low-dose ketamine infusion at 5 mg/kg/h displayed similar behavior to saline controls with the exception of reduced rearing and grooming behavior. A previous investigation reported reductions in fine motor movements and described absent grooming behavior after sub-anesthetic IP ketamine injections [155]. The neural circuitry of rodent grooming behavior is complex and involves multiple brain regions [182]. In our study, ketamine at high-doses induced increased locomotor activity, which may contribute to reduced grooming behavior; however, reduced grooming was also observed at low-doses and reduced

locomotor activity. Thus, reduced grooming observed at both high and low doses of ketamine may not result from altered locomotor activity.

Ketamine 5 mg/kg IV bolus and 20 mg/kg/h infusion impaired PPI but only 10 and 20 mg/kg/h infusions reduced ASR. There are many reports describing PPI interruption in rodents after NMDA antagonist injections [149, 163, 183], which model sensorimotor gating deficits in schizophrenia patients [184, 185]. This investigation confirmed these reports at high-doses, but low-dose ketamine at 2 mg/kg IV bolus and infusions at 5 and 10 mg/kg/h failed to impair PPI (Figures 3 and 6). Ketamine infusions at 10 mg/kg/h and 20 mg/kg/h reduced ASR while both bolus doses and the 5 mg/kg/h infusion showed no effect (Figures 3 and 6). These results confirm previous reports that also found sub-anesthetic ketamine injections had either no change or non-significant reductions in ASR [146, 149, 164]. The ASR reduction following 20 mg/kg/h ketamine infusion was surprising because of its similar plasma ketamine concentration to the high-dose ketamine bolus 5 mg/kg, which had no effect on ASR. It is possible that hyper-locomotive rats throughout the 1-h high-dose ketamine infusion experienced a recovery hypo-locomotive state at the time of ASR measurement, which was 30 min after completion of the infusion. A similar step-wise reduction in ASR was observed for the 5 and 10 mg/kg/h infusions (Figure 2.6), which showed an inverse relationship to locomotor activity (Figure 2.6). Ketamine concentrations in brain tissue were most likely very low at ASR measurement as IV ketamine undergoes rapid metabolism in rat [186]. Similar to PCP, ketamine has dose-dependent receptor affinities [187] where at low brain/blood concentrations, ketamine could preferentially bind to receptors that impact either sensory input or motor output responses and reduce ASR [188].

Intravenous ketamine produced dose-dependent changes in antinociception. The antinociceptive effects of ketamine in various rodent pain models are previously described [169-171, 189], but there is little information regarding duration of antinociception. Ketamine

administered as a 5 mg/kg IV bolus and 10 and 20 mg/kg/h infusions produced long-lasting antinociception (Figure 2.4). Extended duration of ketamine analgesia following infusion stoppage is reported in human literature [190] and in a rodent study that measured opioid/ketamine interaction [170]. Although unbound IV ketamine undergoes rapid metabolism and clearance [186], bound ketamine may prolong NMDA and opioid receptor activation, which may have extended the antinociception observed in the present study. Another possibility is the antinociceptive effect of norketamine, an active metabolite of ketamine [105]. In the present study, norketamine levels increased from 1 to 20 min and essentially plateaued from 20 to 50 min following both IV bolus dosages (Table 2.1); however, norketamine concentrations were greater for the 5 mg/kg bolus and may have contributed to extended antinociception. As a final point, dissociative stereotypy were not observed at the time of nociception measurement, which suggests limited receptor engagement beyond NMDA receptors at the low plasma ketamine concentrations at 20 and 50 min (Table 2.1).

Ketamine and metabolite concentrations in blood following parenteral injections are well-characterized in both rodents [111, 146, 186, 191, 192] and humans [193]. To our knowledge, no studies have described ketamine and norketamine plasma concentrations following a continuous sub-anesthetic IV ketamine infusion in rats. The peak ketamine plasma concentration obtained after 5 mg/kg IV bolus administration (4100 ng/mL) is greater than that reported by Eckert et al. (2015) at the same dose (2800 ng/mL) [192]. Different sampling time points may have contributed to the discrepancy as the peak ketamine sample was collected at 1 min following bolus dosing. The peak plasma concentrations reported for bolus and infusion doses in this study are also greater than those reported in human studies following 0.5 mg/kg IV ketamine infusions for depression treatment [194]. However, there are differences in CYP450 metabolism across species [195], which is the metabolic pathway for ketamine. These inter-species differences

contribute to the larger doses and increased ketamine brain/plasma concentrations required for rodents to yield similar treatment effects compared to humans [192]. Overall, it is anticipated that plasma concentrations described in the present study will provide a reference point for future pre-clinical rodent investigations utilizing IV ketamine infusion and a comparison to previous rodent ketamine models that utilized SC or IP injections.

Intravenous ketamine infusion in rodents is a clinically relevant model that is surprisingly absent from pre-clinical literature. Researchers may prefer to administer ketamine via injections to rodents due to convenience and ease of administration compared to an IV route but injections pose challenges for some neurobehavioral studies. First, ketamine undergoes rapid metabolism and clearance following parenteral injections [111, 196]. Rapid metabolism results in limited brain exposure to ketamine at peak plasma concentration, which is not comparable to the steady state plasma concentration maintained by continuous IV infusions used in clinical practice. Researchers have attempted to mimic ketamine infusions through repeated IP injections [136], but this method results in successive peak and trough plasma concentrations that resemble a saw-tooth profile. Second, injections stress rodents causing increased corticosterone [197] and local tissue necrotic injury possibly due to the acidic pH of ketamine [198]. Repeatedly stressing rodents through manual restraint and painful injections could confound behavioral studies. Lastly, complications are associated with IP injections performed without visual confirmation of needle placement. Inadvertent injections can occur into fat, urinary bladder, muscle, or abdominal organs and result in a 10-20% failure rate [145]. Overall, we suggest that pre-clinical researchers consider using similar ketamine delivery routes used in human treatments, such as IV infusions, to enhance study translation across species.

The present study is not without limitations. Small sample sizes were utilized with rats that underwent repeated exposure to ketamine dosing. For example, ketamine/xylazine

anesthesia was used for the indwelling jugular catheter surgery. However, baseline ketamine plasma concentrations were measured 7-days after surgery and no ketamine was detected in the plasma prior to the experiments. Additionally, rats were given a 4-day period between testing to ensure complete ketamine clearance. Repeated ketamine exposure raises concerns for tolerance of ketamine. However, both bolus and infusion groups underwent a counter-balanced exposure to all dosages and did not display tolerance. These rats showed distinct dose-dependent differences in locomotion, stereotypy, ASR/PPI, and antinociception. Despite these limitations, the study methods also provided some advantages. For example, the use of an operant chamber with metal spring wire and rotating swivel allowed the authors to administer a prolonged ketamine infusion to a freely moving rat. Additionally, the indwelling jugular catheter allowed the study team to conduct repeated *in vivo* blood sampling from rats, reducing the number of rats required for repeated blood sampling.

Lastly, this ketamine investigation utilized only male rats. Previous work has demonstrated sex-related differences in response to anesthetic ketamine doses on metabolism [199] and sub-anesthetic doses on antidepressant-like effects in rats and mice [200, 201]. In fact, there has been an overall trend to exclude female rodents in neuroscience and biomedical research mostly due to unsubstantiated concerns about estrus-cycle mediated hormonal changes [202, 203]. Therefore, the current findings in male rats cannot be generalized to female rats and future investigations regarding the effects of IV ketamine on female animals are warranted.

## 5. Conclusion

IV ketamine administered by bolus and infusion produced dose-dependent changes in dissociative stereotypy, locomotion, sensorimotor gating, and antinociception. Ketamine and norketamine plasma concentrations were described following IV bolus at 1, 20, and 50 min and at the conclusion of a 1-h infusion. The current ketamine infusion model may provide unique

opportunities for future pre-clinical investigations that mirror human ketamine administration. For example, clinicians administer low-dose IV ketamine infusions to treat chronic pain [166], depression [204], and PTSD [117] in humans. Pre-clinical researchers could administer low-dose subanesthetic IV ketamine infusions to rodents that will provide a sustained steady state brain/plasma concentration similar to human applications while limiting dissociative stereotypy. Additionally, there is recent work exploring the effects of an active ketamine metabolite, hydroxynorketamine, as a novel depression treatment [205, 206]. Administering ketamine and metabolite treatments to rodents through an IV infusion model, similar to human investigations, may provide a more clinically relevant route for future translational studies.

**Funding**

**Conflict of Interest**

The authors declare no conflict of interest.

**Disclaimer**

All opinions in this article are those of the authors and do not reflect upon the official policy or position of the Department of the Navy, the Department of Defense, the Uniformed Services University of the Health Sciences, or the United States Government.

# CHAPTER 3

The effects of sub-anesthetic intravenous ketamine infusion on corticosterone and brain-derived neurotrophic factor in the plasma of male Sprague-Dawley rats.

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**Abstract**

Post-trauma anesthetic agents influence neuroendocrine responses that may impact fear-memory. The effects of an intravenous (i.v.) ketamine infusion on mediators of stress and memory in rodents are unknown. Therefore, we used a clinically relevant method to administer a 2h sub-anesthetic i.v. ketamine infusion following a rodent fear-conditioning paradigm (paired tone + foot shock) to evaluate the effects on corticosterone (CORT) and brain-derived neurotrophic factor (BDNF) in the plasma of male Sprague-Dawley rats. We found that sub-anesthetic ketamine infusions (5 and 20 mg/kg/h) dose-dependently increased plasma CORT levels. Ketamine at 20 mg/kg/h significantly reduced plasma BDNF measured 2h after the conclusion of the ketamine infusion. These results demonstrate that a sub-anesthetic i.v. ketamine infusion maintained a heightened neuroendocrine stress response after fear-conditioning and reduced a neurotrophin associated with memory. The behavioral outcomes of these effects are unknown and warrant future investigation.

**IACUC approval:**

The Institutional Animal Care and Use Committee at the Uniformed Services University of the Health Sciences approved this pre-clinical investigation with approval number: PSY-15-829.

## 1. Introduction

Ketamine, a multimodal potent dissociative anesthetic, is often administered in the immediate post-trauma period to provide sedation and analgesia. First responders and clinicians may favor ketamine as a trauma anesthetic due to its cardiovascular stability, maintenance of spontaneous respiration, and high safety ceiling.[6] Likewise, anesthesia professionals may administer peri-operative sub-anesthetic intravenous (i.v.) ketamine infusions to manage acute pain and reduce opioid consumption.[207] However, the effect of immediate post-trauma ketamine administration on mediators of stress and memory are often not considered. Stress hormones expressed following trauma serve to either enhance or impair memory formation based upon their concentration and duration of exposure in the brain.[208] It remains unclear how a prolonged exposure to ketamine, a sympathomimetic agent, may impact the stress response and brain proteins associated with memory formation. Therefore, understanding the effects of ketamine on mediators of stress and memory is an important first step before addressing larger questions related to the potential impact of peri-trauma anesthetics on fear memory and stress related disorders.

Trauma survivors diagnosed with stress related disorders such as post-traumatic stress disorder (PTSD) often suffer from dysfunctional and intrusive fear memories of an adverse event that fail to extinguish.[11] Fear memory, similar to other memory types, is formed and regulated through a series of complex and dynamic stages within the limbic regions of the brain, which include the hippocampus, amygdala, and the prefrontal cortices (PFC).[209] In the hours following trauma, a traumatic memory is consolidated from a short-term labile memory to a long-term stable memory through an enhancement of neural networks within limbic structures.[210] Memory consolidation is of particular interest because it occurs during the post-trauma period when health care providers are administering various pharmaceutical agents that

may impact memory development and storage. Memory consolidation comprises a series of non-linear interactions between various mediators and signaling pathways. In particular, glucocorticoids and brain-derived neurotrophic factor (BDNF) are of significant interest due to their relationship with ketamine.

Corticosterone (CORT; rodents) and cortisol (humans) are a type of glucocorticoid hormone secreted from the adrenal glands that mediate various vital functions in response to a stressor. A particular function of CORT is to regulate memories associated with strong emotions.[211] CORT enters the brain and binds to mineralocorticoid and glucocorticoid receptors within limbic structures,[211] which triggers signaling cascades that either impair or enhance fear memory. The opposing effects of CORT on memory are time, dose, and brain region dependent. Intermediate CORT levels strengthen memory, whereas low and high levels either impair or have no effect on memory consolidation suggesting an inverted U dose relationship.[212, 213] For example, intermediate CORT elevations enhance fear memory consolidation within the amygdala,[212] while either low CORT or prolonged CORT exposure may disrupt memory consolidation in the hippocampus through dendritic atrophy.[214, 215]

There is converging evidence that CORT is one of several signaling molecules that regulate BDNF production.[216] BDNF is a protein required for neuronal growth, neuroprotection, and synaptic plasticity throughout the central nervous system. Altered BDNF levels are associated with dysregulated fear memory disorders such as PTSD.[217] CORT effects BDNF differently depending on the brain region and duration of exposure.[216] Stress induced CORT activation increases BDNF in the amygdala,[218] which promotes neuronal growth and contributes to the enhanced emotional valence associated with fear memory. Conversely, reduced BDNF in the PFC and hippocampus leads to neuronal atrophy, impaired memory, and disrupted fear extinction, which is a hallmark characteristic of PTSD.[219, 220] BDNF can also

cross the blood-brain-barrier[221] allowing researchers to use peripheral blood BDNF levels as a potential biomarker for stress related disorders.[222] Additionally, blood BDNF is positively correlated with brain BDNF concentrations in rodents, suggesting blood sampling as a potential non-invasive method for *in vivo* monitoring of neuronal health.[223]

Sub-anesthetic ketamine administration directly impacts both CORT and BDNF. Low-dose i.v. ketamine infusions increase cortisol in blood and saliva in humans.[224] Similarly, a single intraperitoneal (i.p.) injection or i.v. ketamine bolus increases CORT in rodents.[225, 226] However, what remains unknown is the effect of a prolonged sub-anesthetic i.v. ketamine infusion administered immediately after trauma on the plasma CORT response, which could mediate fear memory consolidation associated with trauma. Effects of ketamine on BDNF, a protein associated with memory formation, are less clear. A single, low-dose (10 mg/kg) ketamine i.p. injection increases BDNF in rodent depression models,[119] while chronic ketamine administration reduces BDNF production in both humans and rodents.[227, 228] Similar to CORT, the effect of a sub-anesthetic i.v. ketamine infusion on plasma BDNF protein concentration after trauma remains unclear.

A major limitation of rodent behavioral research is ketamine delivery via i.p. injections into the abdomen, which may hinder translation to human investigations and clinical practice that utilize i.v. infusions. A one-time ketamine i.p. injection in rodents results in a short duration of action secondary to a short drug half-life.[146] On the other hand, an i.v. infusion maintains a steady-state drug-plasma concentration over an extended time interval allowing for maximum impact on neurobiological mediators such as CORT and BDNF.

Clinically relevant medication delivery routes are poorly utilized in rodent models that aim to characterize drug effects on fear memory mechanisms. The effects of immediate post-trauma sub-anesthetic i.v. ketamine infusions on the stress response and brain proteins associated

with fear memory are unknown. Therefore, the purpose of our investigation was to determine the effects of sub-anesthetic i.v. ketamine infusions following fear conditioning on CORT and BDNF, which are mediators of stress and memory. Based upon previous studies, we hypothesized that a sub-anesthetic i.v. ketamine infusion may increase both CORT and BDNF levels in the plasma of rats.

## 2. Materials and Methods

*2.1 Animals.*

Adult male Sprague-Dawley rats (Envigo Laboratories; Dublin, VA) weighing 250-300g were housed individually in clear Plexiglas shoebox cages in a climate controlled environment with food/water *ad libitum*. Animals were habituated to a 12-h reversed dark:light cycle (lights off at 0600; testing during dark cycle) and handled daily for 7 days prior to testing. All procedures were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of the Health Sciences (Bethesda, MD).

*2.2 Intravenous Catheter.*

A jugular venous catheter (3Fr, polyurethane; Instech, Plymouth Meeting, PA) was surgically placed under isoflurane anesthesia by personnel at Envigo Laboratories prior to animal arrival. The catheter was tunneled under the skin and connected to a vascular access button (Instech) that exited the dorsal position between the front rodent scapulae. All rodents remained ketamine naïve throughout surgery and post-operative care following catheter placement. Catheters were flushed every three days to verify venous patency and locked with 0.1mL heparin/glycerol solution (Braintree Scientific; Braintree, MA).

*2.3 Grouping.*

Animals were randomly assigned to groups (n = 10-12) as follows: Group 1 (no fear conditioning and saline infusion), Group 2 (no fear conditioning and ketamine bolus + 20 mg/kg/h infusion), Group 3 (fear conditioning and saline infusion), Group 4 (fear conditioning and ketamine bolus + 5 mg/kg/h infusion), and Group 5 (fear conditioning and ketamine bolus + 20 mg/kg/h infusion).

*2.4 Fear conditioning and Apparatus.*

The fear conditioning box had a metal grid floor surrounded by Plexiglas walls and was dimly lit by a single house light (2-3 lux). The fear conditioning box was housed inside a larger sound-attenuating chamber with a background noise level of 55 dB (Coulbourn Instruments, Lehigh Valley, PA). Groups 3, 4, and 5 underwent auditory cued fear conditioning as previously described [229] at time 0800. After a three-minute acclimation period, rats were presented with three pairings of an auditory tone (5kHz, 75dB, 20s) that co-terminated with a mild foot shock (0.8mA, 0.5s). There was an intertrial interval of 90-120s to prevent tone prediction. Fear conditioned rats were removed 60s after the final tone-foot shock pairing. Groups 1 and 2 were placed into the chamber and underwent the same sequence of events at time 0800, but were exposed to the tone without foot shock and thus, did not undergo fear conditioning.

*2.5 Ketamine Infusion.*

Immediately after fear conditioning, each rat was placed into an operant conditioning chamber (Med Associates Inc., St. Albans, VT). Each chamber was equipped with an infusion pump (Harvard Pump 11 Elite, Holliston, MA) using a 10-mL syringe connected to a fluid swivel (Instech) by polyurethane tubing encased in a metal spring wire tether to prevent chewing and tubing entanglement. The tether was attached to the vascular access button on the rat using a luer-lock connection. All tethered rats had free mobility within the chamber during saline or

ketamine infusions. A dim red-light illuminated each box to facilitate observation by the study team.

*2.6 Ketamine Dosing.*

Racemic (+/-) ketamine hydrochloride (100 mg/mL) (Mylan Institutional LLC, Rockford, IL) was diluted in 0.9% sterile saline prior to dosing. Groups 2 and 5 received a ketamine (5 mg/kg, i.v.) bolus and then a 2h ketamine (20 mg/kg/h, i.v.) infusion. Group 4 received a ketamine (2 mg/kg, i.v.) bolus and then a 2h ketamine (5 mg/kg/h, i.v.) infusion. These sub-anesthetic i.v. ketamine bolus and infusion doses were previously determined in our laboratory [230]. Groups 1 and 3 received a saline bolus and 2h saline infusion (1 mL/h). All ketamine and saline bolus doses were delivered in a 1 mL/kg volume.

*2.7 Blood Sampling.*

Baseline blood samples were taken 1 day prior to fear conditioning at consistent times (time 1000), 2h after fear conditioning (the conclusion of the ketamine or saline infusion – time 1000), and 4h after fear conditioning (2h after the end of the infusion – time 1200) (Figure 1). Blood was collected *in vivo* from the implanted catheter at the baseline and at the 2h time point. Blood (0.2 mL) was withdrawn to clear the catheter of medication and prime with fresh blood. Then 0.3 mL of blood was withdrawn and placed into 1.5 mL Eppendorf tubes with 10 µL of ethylene-diamine-tetra-acetic-acid (EDTA) additive to prevent blood clotting. Trunk blood was collected following decapitation at the 4h time point and placed in EDTA tubes. Blood samples were centrifuged at 4,000 rpm x 20 min at 4°C (Eppendorf 5415 R). Plasma supernatant was collected and stored at -80°C for future analysis.
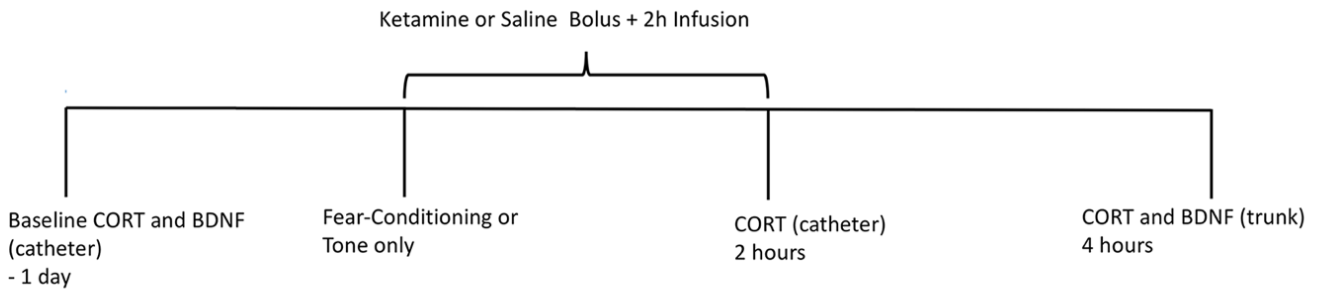
Figure 3.1. **Experimental timeline.** All blood samples were collected as plasma. Baseline and 2h samples were collected from an implanted jugular venous catheter *in vivo*. The sample collected at 4h was from trunk blood. Abbreviations: CORT, corticosterone; BDNF, brain-derived neurotrophic factor.

*2.8 CORT Assay.*

CORT was quantified using a DetectX® corticosterone enzyme-linked immunosorbent assay (ELISA) kit (Arbor Assays; Ann Arbor, MI) per manufacturer protocol. Plasma samples (10 µL) were combined with dissociation reagent (10 µL) then diluted to 1:100 with assay buffer. Standards were prepared in serial dilutions (10,000 to 78.125 pg/mL). Next, standards or samples (50 µL) were added to each well in duplicate using a 96-well plate. Then, CORT conjugate (25 µL) and antibody (25 µL) were added to the samples. After a 1h incubation at room temperature on a plate shaker, the wells were aspirated and washed three times with wash buffer (300 µL). Then, TMB (3,3', 5,5' – tetramethlybenzidine) substrate was added per well (100 µL) and allowed to incubate for 30-minutes. Finally, stop solution (50 µL) was added to each well and the plate optical density was measured at 450 nm (200 Pro Microplate Reader, Tecan) and calculated as ng/mL.

*2.9 BDNF Assay.*

Plasma BDNF was quantified using an Emax® Immunoassay System (ELISA) (Promega; Madison, WI) according to manufacturer protocol. The well bottoms of a 96-well plate were coated with anti-BDNF monoclonal antibody and incubated at 4°C overnight. Standards were

prepared in serial dilutions (500-7.8 pg/mL) and plasma samples were diluted 1:4 in buffer.

Wells were blocked with Block & Sample buffer for 1h and washed with Tri-buffered saline

containing 0.1% Tween 20 (TBST). Next, samples or standards (100 µL) were added to each

well in duplicate and incubated for 2h on a plate shaker. After TBST washing, plates were

incubated for 2h with Anti-Human BDNF polyclonal antibody (100 µL). Then, after TBST

washing, plates were incubated for 1h with Anti-IgY HRP (100 µL). After a final TBST

washing, plates were incubated for 10 min with TMB One solution (100 µL) and the reaction

was stopped with 100 µL 1N hydrochloric acid. The optical densities of standards and samples

were measured at 450 nm (200 Pro Microplate Reader, Tecan) and calculated as pg/mL. BDNF

Emax® Immunoassay System sensitivity is 15.6 pg/mL.

*2.10 Statistical Analysis.*

All data are presented as mean ± standard error of the mean (SEM) and were analyzed using

GraphPad Prism (GraphPad Software, version 7.0). Repeated-measures two-way analysis of

variance (ANOVA) with Time and Ketamine as factors, one-way ANOVA, and unpaired t-tests

were used for CORT and BDNF assays as appropriate. Bonferroni post-hoc tests were used to

compare significant ANOVA differences. BDNF data were normalized to the non-fear

conditioned saline control group. The accepted level of significance was $p < 0.05$.

## 3. Results

*3.1 CORT.*

We compared the effect of a 2h ketamine infusion on CORT concentrations in plasma at

three time-points (baseline, 2h, and 4h) in both non-fear conditioned and fear conditioned rats

using treatment and time as factors (Figure 2). There were no differences between groups at

baseline. First, we compared non-fear conditioned rats and found significant main effects of

Treatment $F$ (1,22) = 35.48 ($p < 0.001$), Time $F$ (2,44) = 60.57 ($p < 0.001$), and a Treatment x

Time interaction $F$ (2,44) = 35.01 ($p < 0.001$). Post-hoc testing showed that ketamine infusion

(20 mg/kg/h) induced a significant increase in plasma CORT concentration (568.1 ± 70.81

ng/ml) compared to a saline vehicle at 2h (114 ± 17.85 ng/ml; $p < 0.001$) (Figure 2a). Next, we

compared fear conditioned rats and found significant main effects of Treatment $F$ (2,33) = 19.87

($p < 0.001$), Time $F$ (2,66) = 92.38 ($p < 0.001$), and a Treatment x Time interaction $F$ (4,66) =

19.63 ($p < 0.001$). Post-hoc testing revealed that ketamine infusions at 5 mg/kg/h (405 ± 41.03

ng/mL) and 20 mg/kg/h (731 ± 106.5 ng/mL) dose-dependently increased CORT concentrations

compared to the saline vehicle at 2h (122 ± 25.04 ng/mL; $p < 0.001$) (Figure 2b). Saline

infusions induced mild elevations in CORT at 2h in both fear and non-fear conditioned rats

compared to baseline values, but these differences were not significant. Additionally, we

observed depressed CORT concentrations at 4h compared to baseline measures, but these

differences were also not significant.  Lastly, we compared fear conditioned to non-fear

conditioned groups at each time point (baseline, 2h, and 4h). There were no differences in CORT

concentration levels between fear conditioned and non-fear conditioned groups at the baseline or

4h time points. Additionally, there were no significant differences between the ketamine (20

mg/kg/h) infusion in the non-fear conditioned group compared to ketamine (5 and 20 mg/kg/h)

infusions in the fear conditioned group.
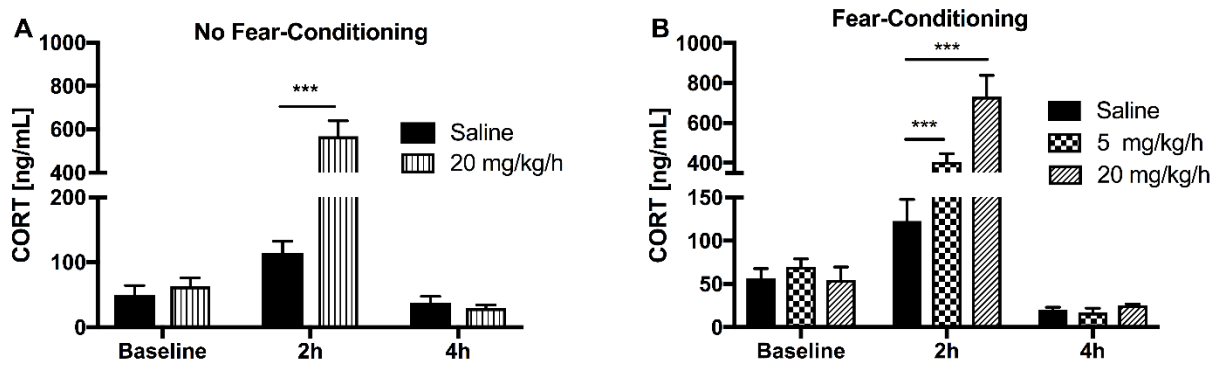
Figure 3.2. **Effect of ketamine infusion on corticosterone (CORT).** CORT was measured at the baseline, immediately after the infusion (2h), and 2h after the infusion (4h). A) Ketamine infusion (20 mg/kg/h x 2h) significantly increased CORT concentration in rats that did not undergo fear conditioning. B) Ketamine infusions (5 & 20 mg/kg/h x 2h) dose-dependently increased CORT concentrations compared to a saline infusion among rats that experienced fear conditioning. Data are shown as mean $\pm$ SEM (*** $p < 0.001$, n = 10-12 per group).

*3.2 BDNF.*

We compared plasma BDNF concentrations at a baseline and 2h after a ketamine infusion (4h) in both non-fear conditioned and fear conditioned rats (Figure 3). Data are reported as normalized BDNF values to the non-fear conditioned saline control. There were no significant group differences in BDNF at the baseline. First, we compared non-fear conditioned rats and found that ketamine infusion at 20 mg/kg/h (0.44 $\pm$ 0.06) induced a significant reduction in plasma BDNF concentrations compared to the saline vehicle (1$\pm$ 0.16; $t$ (3.248), $p < 0.01$, two-tailed) measured at 4h (Figure 3a). Next, we compared fear conditioned rats and found significant differences between the ketamine infusions and the saline vehicle $F$ (2,32) = 3.89 ($p < 0.05$). Post-hoc testing revealed that a ketamine infusion at 20 mg/kg/h significantly reduced BDNF concentration (0.65 $\pm$ 0.13) compared to saline at 4h (1.2 $\pm$ 0.15; $p < 0.05$) (Figure 3b).

Although the ketamine infusion at 5 mg/kg/h also showed a reduction in BDNF (0.75 ± 0.16), the result was not statistically significant.
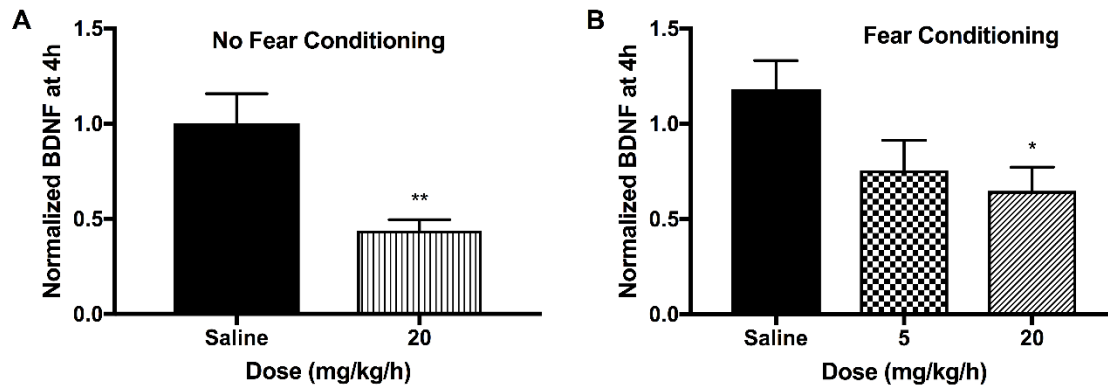


Figure 3.3. **Effect of ketamine infusion on brain-derived neurotrophic factor (BDNF).** BDNF was measured 2h after the conclusion of a ketamine infusion. A) Ketamine infusion (20 mg/kg/h x 2h) significantly decreased plasma BDNF concentration in rats that did not undergo fear conditioning. B) Ketamine infusion (20 mg/kg/h x 2h) significantly reduced plasma BDNF concentration among rats that experienced fear conditioning. Data are shown as mean ± SEM (* $p < 0.05$, ** $p < 0.01$, n = 10-12 per group).

## 4. Discussion

Although clinicians typically administer ketamine to humans using the i.v. route, most pre-clinical researchers administer ketamine via i.p. injections to rodents, which limits translational value to clinical practice. There are no published reports, to our knowledge, that have measured the effects of a sub-anesthetic i.v. ketamine infusion administered immediately after trauma on biomarkers of stress and fear memory. Therefore, we administered a sub-anesthetic i.v. ketamine infusion immediately after fear conditioning and measured effects on plasma CORT and BDNF levels in rats.

Sub-anesthetic i.v. ketamine infusions (5 and 20 mg/kg/h) dose-dependently increased CORT levels in rats (Figure 2). These results are in line with previous investigations that observed elevated cortisol in the blood and saliva of humans following sub-anesthetic i.v.

ketamine infusions (0.29-0.57 mg/kg over 40-60 min).[9, 224] Similar CORT elevations were

observed following anesthetic and sub-anesthetic ketamine i.p. injections (15-120 mg/kg) and

single i.v. bolus doses (2 and 35 mg/kg) in rodents.[225, 226, 231] Some investigators attribute

the sympathomimetic properties of ketamine to trigger the increased CORT response,[9] which

suggests a direct action on the adrenal gland. In contrast, other studies reported no CORT effects

when ketamine was administered to rats without a pituitary gland, which infers ketamine must

act either at the hypothalamus or pituitary and not directly at the adrenal gland.[226] Similarly,

ketamine added to isolated adrenal cells *in vitro* had no direct effect on CORT, while ketamine

increased the expression of adrenocorticotropic hormone (ACTH) when added to isolated

anterior-pituitary cells.[232] Additionally, the hypothalamic neuroendocrine circuitry is rich in

*N*-methyl-*D*-aspartate (NMDA) receptors, the principal receptor type for ketamine effects. Taken

together, these data provide evidence that an i.v. ketamine infusion increases CORT levels

through direct hypothalamic and/or pituitary stimulation and not through direct action at the

adrenal glands.

Elevated CORT levels following trauma in humans, as well as fear conditioning in

rodents, may impact fear memory consolidation. An inadequate glucocorticoid-stress response

following trauma may disrupt the information processing and predict PTSD

symptomatology.[233] Zohar et al. reported that high-dose hydrocortisone administered during

emergency care following a traumatic event reduced core symptoms of PTSD up to 3-months

post-trauma.[234] Similarly, CORT elevations induced by acute immobilization stress to rodents

after fear conditioning reduced subsequent fear memory.[235] In contrast, other studies have

reported increased CORT expression during memory consolidation to enhance fear memory,

which is an adverse effect.[69, 213] The adverse effects of acute CORT are supported by other

studies, which described fear memory consolidation as dependent upon acute CORT elevations

following a stressor.[211, 212] CORT binds to glucocorticoid receptors within fear memory structures such as the amygdala and hippocampus following trauma exposure to signal the production of proteins that enhance memory consolidation.[211] The memory enhancing effects of CORT also require simultaneous noradrenergic activity.[212] Interestingly, ketamine is a sympathomimetic agent that also increases the CORT response. Therefore, it is plausible that our sub-anesthetic i.v. ketamine infusion administered immediately after fear conditioning may serve to enhance fear memory through CORT and sympathomimetic synergistic mechanisms.

Sub-anesthetic i.v. ketamine infusions (5 and 20 mg/kg/h) decreased plasma BDNF levels in rats (Figure 3). These results were not anticipated as they contradicted previous investigators that reported increased BDNF following single sub-anesthetic i.p. ketamine injection (10-15 mg/kg, i.p.) in rodent depression models.[236] Our results, however, are in line with other studies that observed reduced blood BDNF among chronic ketamine abusers[228] and reduced brain BDNF among rats that received chronic ketamine (25 mg/kg, i.p.) injections over seven days.[227] Similarly, chronic sub-anesthetic ketamine (25 mg/kg, i.p.) injections also reduced brain BDNF in a rodent model of schizophrenia.[237]

The mechanisms by which ketamine increases BDNF in rodent depression models are well studied and a variety of receptors and signaling pathways are implicated. It is generally proposed that ketamine antagonizes the NMDA receptor on pre-synaptic γ-aminobutyric acid-ergic (GABAergic) neurons. NMDA blockade disinhibits pre-synaptic GABAergic neurons leading to a paradoxical increase in pre-synaptic glutamate release.[238] Glutamatergic signaling culminates in the post-synaptic production of BDNF via activation of the mammalian target of rapamycin (mTOR) pathway.[119]

On the other hand, prolonged NMDA blockade of pre-synaptic GABAergic neurons can induce excessive glutamate release and negative consequences. Hyperactive glutamate leads to

the dysfunctional influx of calcium and sodium ions that trigger excitotoxicity and neuronal apoptosis.[239] Furthermore, glutamatergic dysregulation is implicated in a variety of psychiatric diagnoses, including PTSD.[240] The seemingly opposite effects of ketamine on BDNF may be related to the duration of the NMDA receptor blockade. A one-time, low-dose ketamine i.p. injection administered in rodent depression models appear to increase BDNF,[236] while repeated/chronic ketamine in humans and other rodent models reduce BDNF.[227, 228] The sub-anesthetic i.v. ketamine infusions administered in our investigation may exert a similar effect to that of chronic ketamine paradigms by antagonizing NMDA receptors over a sustained time interval possibly leading to a dysregulated glutamate/BDNF mechanism and our reduced plasma BDNF results.

The blunted response of BDNF observed in our investigation stimulates intriguing possibilities. BDNF is necessary to form long-term memories, while reductions of BDNF can hinder various types of memory formation including fear memory.[241] Disrupting fear memory consolidation could weaken fear memories and potentially prevent stress related disorders. However, there is evidence that inadequate BDNF formation and reduced synaptic plasticity can dysregulate fear memory formation.[234] Impaired BDNF production, secondary to a BDNF gene mutation (BDNF val66met), may predispose certain individuals to develop PTSD and anxiety disorders, while also impairing fear-extinction.[242] Nevertheless, manipulating BDNF levels in either direction after trauma may impact fear memory formation and processing and needs further investigation.

Overall, the clinical significance of our findings is difficult to interpret in the absence of behavioral data. Ketamine is a popular trauma anesthetic and sub-anesthetic i.v. ketamine infusions are used in the peri-operative period to manage acute pain and minimize opioid consumption. However, the long-term psychological impacts of the immediate post-trauma

administration of ketamine are unclear. Human subject investigations on post-trauma ketamine are scant and with mixed results. McGhee et al. found no relationship between intraoperative ketamine administration and PTSD risk among burn patients, [131] however, these ketamine doses were given significantly after the initial trauma and memory consolidation period. In contrast, Winter and Irle reported increased rates of PTSD among burn patients who received 12-days of benzodiazepine/ketamine analgesia after injury.[134] Schonenberg et al. administered ketamine in the immediate period after trauma and reported an increase in acute stress disorder and core symptoms of PTSD compared to patients that received opiate and non-opiate analgesics.[133] Anesthetic and sub-anesthetic ketamine i.p. injections initiated during the fear memory consolidation phase in rodents have also produced mixed results showing either improvement or no change in fear behaviors.[136, 243] However, more recent investigations have shown ketamine administered after a rodent stressor to worsen fear memory and impair social interaction, which are characteristics of PTSD-like behavior.[137, 138, 244]

We caution clinicians not to compare our findings to those of recent investigations that have used ketamine to temporarily reverse depressive and PTSD symptoms. Timing of ketamine administration could produce different results. We contend that a sub-anesthetic ketamine infusion administered immediately after trauma may impact fear memory acquisition and consolidation through dose-dependent alterations of stress and memory mediators. In contrast, ketamine administered months to years later as a treatment may impact different memory processes such as retrieval and reconsolidation and prove beneficial.

Our investigation is not without limitations. We used only male rats and therefore, we cannot generalize our results to female rats. There are gender related differences in response to stress and anxiety that require further investigation. Since we measured BDNF in plasma, we do not know the brain region-specific expression of BDNF. Regional differences in BDNF

expression is an area for future investigation. Clinically, a benzodiazepine is often co-administered with ketamine to offset the experience of dissociative symptoms. We did not administer a benzodiazepine in our rodents as we wanted to examine ketamine specific effects without a confounding psychoactive medication. Interestingly, benzodiazepines are GABA agonists that may blunt the potential hyper-glutamatergic and excitotoxic effects of a prolonged ketamine infusion. Lastly, we did not measure behavioral effects because the biomarker results from this study were used to support a grant proposal for a future investigation that will explore sub-anesthetic ketamine infusion effects on fear memory and behavior.

In conclusion, sub-anesthetic i.v. ketamine infusions administered after rodent fear conditioning dose-dependently increased CORT and reduced plasma BDNF. These findings illustrate the impact of intravenous anesthetic agents administered after trauma on mediators of stress and memory. Alterations of these mediators may have long term unintended psychological consequences. For instance, we do not know what effect, if any, elevated CORT levels for an extended time period after trauma may have on memory and emotional processing of a traumatic event. To that end, we strongly encourage future studies that will extend our CORT and BDNF results and examine the effects of post-trauma sub-anesthetic i.v. ketamine infusions on fear memory and stress related disorders.

# CHAPTER 4

The effect of immediate and delayed sub-anesthetic intravenous ketamine infusion on activity, fear memory retrieval, extinction, and renewal in male Sprague-Dawley rats.

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**Abstract**

Injuries are the most common traumatic event worldwide, and those who survive traumatic injury are twice as likely to develop post-traumatic stress disorder (PTSD) compared to those without physical injury. First responders and clinicians administer ketamine, a multimodal dissociative anesthetic, in the immediate aftermath of traumatic injury to provide sedation and analgesia for trauma victims. Although a seemingly ideal trauma anesthetic, ketamine is also a psychomimetic agent that induces hallucinations and delusions. The impact of post-trauma ketamine on fear memory and the development of stressor related disorders is not clear. Moreover, there is an absence of pre-clinical literature that utilizes a clinically relevant intravenous (i.v.) ketamine infusion model in rodents. Therefore, in the present study, we administered a 2-h sub-anesthetic i.v. ketamine infusion at varying dosages (0, 1, 5, and 10 mg/kg/h) either immediately or 1-d after rodent fear conditioning (auditory tone + footshock) and measured effects on locomotor activity, fear memory retrieval, extinction, and renewal. Both immediate (5 and 10 mg/kg/h) and delayed (5 mg/kg/h) i.v. ketamine infusions dose-dependently suppressed vertical (rearing) and horizontal activity. Immediate and delayed ketamine infusions (5 mg/kg/h) enhanced fear memory retrieval and delayed fear extinction. An immediate ketamine infusion (5 mg/kg/h) enhanced fear renewal to context and cue presentations, while a delayed infusion (5 mg/kg/h) enhanced renewal to context and not cue. These results indicate that sub-anesthetic i.v. ketamine infusion in the post-trauma period may dose-dependently impact fear memory processes associated with trauma and stressor related disorders.

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# 1. Introduction

Accidents and injuries are the most common trauma exposures worldwide [245] and survivors of traumatic physical injury are twice as likely to develop post-traumatic stress disorder (PTSD) compared to those without physical injury [246]. First responders and clinicians administer ketamine, a multimodal dissociative anesthetic, to the traumatically injured to provide sedation and analgesia without compromising hemodynamic stability and spontaneous respirations [6]. Clinicians may also administer ketamine in the post-operative period via continuous intravenous (i.v.) infusion at sub-anesthetic dosing to alleviate acute pain and reduce opioid consumption [207]. Despite apparent advantages as a trauma anesthetic, ketamine is also a psychomimetic agent that induces hallucinations, delusions, and dissociation [9, 10], which raise concerns regarding adverse psychological outcomes following trauma exposure. Trauma survivors diagnosed with PTSD often suffer from intrusive fear memories that fail to extinguish [11-13] secondary to a dysfunctional glutamatergic signaling system [104]. Since ketamine is known to dysregulate glutamate signaling through *N*-methyl-*D*-aspartate (NMDA) antagonism [238], it is important to understand the impact of post-trauma ketamine administration on fear memory and stress related disorders.

Human PTSD investigations exploring post-trauma ketamine administration have shown mixed results. McGhee et al. conducted a retrospective review of United States service members who suffered severe combat related burns and received intraoperative ketamine administration at a stateside facility after initial injury [131]. They found no relationship between ketamine administration and a PTSD diagnosis; however, they did not report elapsed time from combat injury to ketamine administration, co-administered psychoactive medications, or co-morbid injuries associated with PTSD such as depression or mild traumatic brain injury. In contrast, Schonenberg et al. administered ketamine analgesia immediately after traumatic physical injury.

Patients who received ketamine reported increased dissociation, acute stress disorder (ASD), and PTSD symptoms compared to those that received opioid and non-opioid analgesics [133]. These results support an earlier investigation that described increased rates of PTSD among burn patients who received 12-days of benzodiazepine/ketamine analgesia after injury [134].

Pre-clinical investigations exploring the effects of ketamine on fear behaviors in rodents are also limited and have mixed results. Ketamine administered via intraperitoneal (i.p.) injections at anesthetic and sub-anesthetic dosages following rodent PTSD models have either improved [243], worsened [137, 138, 247], or had no effect [136] on fear behaviors in rodents. Ketamine effects on rodent fear extinction are also mixed. Mice that received a single ketamine i.p. injection 1-h or 1-week after fear conditioning showed no change [141] while ketamine injected 1-d after fear conditioning in rats improved extinction learning [140]. Mixed results among fear memory and extinction investigations are not surprising considering the varying ketamine dosages, administration times, differing rodent PTSD models, and variations in rodent species. Moreover, each of the studies utilized i.p. injections to deliver ketamine, which may limit translation to human investigations and clinical practice where ketamine is widely administered via i.v. infusions.

Although a convenient and expedient route of administration in rodents, ketamine i.p. injections cannot maintain a steady state drug plasma concentration similar to an i.v. infusion. Following an injection, ketamine plasma concentrations rapidly peak and decline secondary to drug distribution and elimination [111, 146, 186]. In comparison, an i.v. infusion maintains an elevated ketamine plasma concentration over an extended time period [230], which allows for maximal impact on neurobiological mechanisms. Pre-clinical literature that explores post-fear conditioning effects of ketamine on fear memory and fear extinction are limited and, to our knowledge, there are no fear memory investigations that administer ketamine via the clinically

relevant i.v. infusion route to rodents. Therefore, the primary aim of the current study was to investigate the dose-dependent effects of sub-anesthetic i.v. ketamine infusions administered immediately after fear conditioning on subsequent fear memory retrieval, extinction, and renewal. In order to rule out ketamine effects on fear memory consolidation, we also investigated the effects of a delayed i.v. infusion administered 1-d after fear conditioning on the same fear memory processes.

## 2. Materials and methods

### 2.1 Animals

Adult male Sprague-Dawley rats (Envigo Laboratories; Dublin, VA) weighing 300 - 350 g were used for all experiments. Rats were single housed in clear Plexiglas shoebox cages in a climate controlled environment with food/water *ad libitum*. Animals were habituated to a 12-h reversed dark/light cycle (off at 0600; testing during dark cycle) and handled daily for 7-d prior to testing. All procedures were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of the Health Sciences (Bethesda, MD).

### 2.2 Intravenous Catheter

A jugular venous catheter (3Fr, polyurethane; Instech, Plymouth Meeting, PA) was surgically placed under isoflurane anesthesia by personnel at Envigo Laboratories prior to animal arrival. The catheter was tunneled under the skin and connected to a vascular access button (Instech; Plymouth Meeting, PA) that exited the dorsal position between the front rodent scapulae. All rodents remained ketamine naïve throughout surgery and post-operative care following catheter placement. Catheters were flushed every three days to verify venous patency and locked with 0.1 mL heparin/glycerol solution (Braintree Scientific; Braintree, MA).

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## 2.3 Fear Conditioning Apparatus and Procedure

Fear conditioning was carried out in a chamber (context A) constructed with Plexiglas and aluminum walls (Coulbourn Instruments; Lehigh Valley, PA) on Day 0 (Figure 4.1). The chamber contained a metal grid floor and was dimly lit by a single house light (2-3 lux). All rods were wired to a shock generator (Coulbourn Instruments) and a wall-mounted speaker provided the auditory stimuli. A computer interface program controlled the delivery of footshock and auditory stimuli. A small infrared video camera mounted above the chamber facilitated video recording and observation. The chamber was housed inside a larger sound-attenuating box (Coulbourn Instruments) with a background noise level of 55 dB.

Rats underwent a fear conditioning procedure as previously described [229]. After a 180-s acclimation period, rats received three pairings of an auditory tone (5 kHz, 75 dB, 20-s) that co-terminated with a mild footshock (0.6 mA, 1-s). There was an inter-tone interval (ITI) that ranged from 90 - 120-s to prevent tone prediction. Rats were removed 60-s after the final tone-footshock pairing and received either an immediate i.v. ketamine infusion or a delayed i.v. ketamine infusion 1-d later (Figure 4.1). The conditioning chamber was cleaned with 70% ethanol and thoroughly dried after each use.

## 2.4 Ketamine Infusion and Activity

Racemic (+/-) ketamine hydrochloride (100 mg/mL) (Mylan Institutional LLC; Rockford, IL) was diluted in 0.9% sterile saline and was administered i.v. in an operant conditioning chamber (Med Associates Inc.; St. Albans, VT). Each chamber was equipped with an infusion pump (Harvard Pump 11 Elite; Holliston, MA) using a 5 mL glass syringe (Hamilton) connected to a fluid swivel (Instech; Plymouth Meeting, PA) by polyurethane tubing encased in a metal spring wire tether to prevent chewing and tubing entanglement. The spring-wire tether was attached to the vascular access button on the rat using a luer-lock connection. All tethered rats had free

mobility within the chamber during the infusion. A dim red-light illuminated each box to facilitate observation by the study team.

The drug infusion chamber was equipped with 2 infrared photo beams that counted horizontal activity during the drug infusion period. The number of beam breaks were recorded throughout the 2-h infusion and grouped into 20-min bins (6 bins total). To measure vertical activity, a study member, blind to dosing, observed and counted the frequency of rearing episodes over the final 10-min of the 2-h infusion. A rearing episode was defined as standing on the hind legs with or without the support of the front paws on a wall. Total number of rearing episodes were tallied and compared between the groups.

*2.5 Drug Dosing*

*2.5.1 Immediate Ketamine*

Rats were randomly assigned to 4 groups (n = 9-11/group) which included a saline control group and ketamine dosing groups at 1, 5, and 10 mg/kg/h. Ketamine groups received a ketamine i.v. bolus and 2-h i.v. infusion immediately after fear conditioning on Day 0 (Figure 4.1A). Bolus ketamine doses were 0.5 mg/kg for the 1 mg/kg/h group and 2 mg/kg for the 5 and 10 mg/kg/h groups. The sub-anesthetic i.v. ketamine bolus and infusion doses were determined based on our previous study [230]. The saline control group received a bolus and 2-h saline infusion (1 mL/h) immediately after fear conditioning. All ketamine and saline bolus doses were delivered in a 1 mL/kg volume.
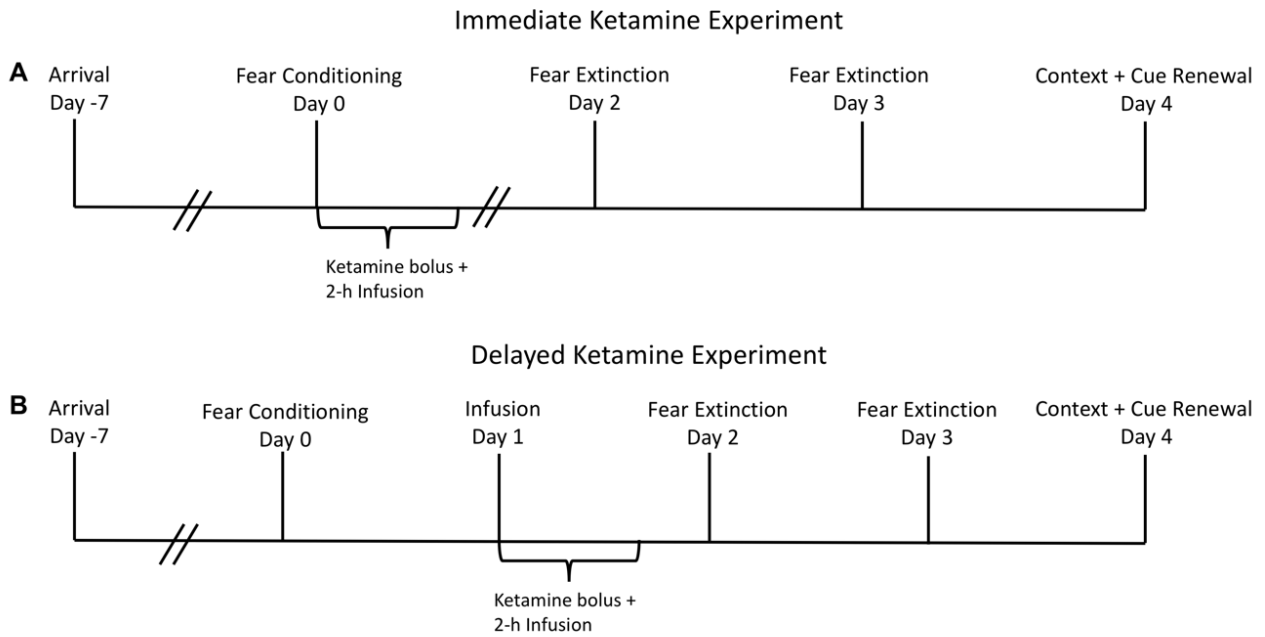
Figure 4.1. **Experimental design.** A) Immediate ketamine experiment. A 2-h sub-anesthetic i.v. ketamine infusion at 1, 5, or 10 mg/kg/h was administered immediately after fear conditioning (Context A) on Day 0. Fear extinction testing consisted of 12 tone trials on Days 2 and 3 in novel Context B. Contextual and Cued fear memory renewal occurred in Context A on Day 4. B) Delayed ketamine experiment. A 2-h sub-anesthetic i.v. ketamine infusion at 5 mg/kg/h was administered 1-d after fear conditioning on Day 1. Rats underwent the same fear extinction testing (Days 2 and 3) and renewal (Day 4) as described in the immediate ketamine experiment.

## 2.5.2 Delayed Ketamine

Based on the immediate ketamine experiment, we identified the infusion dose 5 mg/kg/h as an optimal dose affecting fear memory in rats. The goal of this experiment was to test whether a delayed ketamine infusion after fear conditioning also affects fear memory behaviors. Rats were randomly assigned into two groups (n = 8/group) that received either a saline or ketamine i.v. bolus and infusion 1-d after fear conditioning (Fig.1B). The ketamine group received a bolus (2 mg/kg i.v.) and a 2-h infusion (5 mg/kg/h), while the saline control group received a saline bolus and infusion (1 mL/h). The ketamine and saline boluses were delivered in a 1 mL/kg volume.

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*2.6 Fear Memory Retrieval and Extinction*

Fear retrieval and extinction testing were carried out in a separate chamber (context B) constructed with Plexiglas and aluminum walls (Coulbourn Instruments; Lehigh Valley, PA) on Days 2 and 3 (Figure 4.1). The chamber consisted of altered geometry (slanted Plexiglas ceiling), altered spatial cues (red and black tape on walls), a plastic floor covered with a thin layer of shredded wood chip bedding, and a novel odorant (1% acetic acid). A small house light illuminated the chamber and a mounted infrared video camera facilitated video recording and observation. A wall-mounted speaker delivered the auditory stimuli. Context B was housed inside a larger sound-attenuating box (Coulbourn Instruments) with a background noise level of 55 dB.

Rats underwent fear retrieval and extinction testing as previously described [140]. After an acclimation period of 180-s, rats received 12 auditory tone presentations (5 kHz, 75 dB, 20-s; ITI 90 - 120-s) in context B. An observer blind to the treatment groups measured the freezing time during the delivery of the tone (20-s) using a stopwatch. Freezing was defined as no body movement except as required for respiration and was expressed as a percent freezing during the 20-s tone. Freezing data were averaged and presented in blocks of 2 trials (6 blocks in total).

*2.7 Fear Memory Renewal*

Contextual and cued fear renewal were tested in context A on Day 4 (Figure 4.1). Rats were placed in context A and freezing (contextual fear) was measured in the period of 180-s. At the conclusion of the 180-s observation period, a single auditory cue (5kHz, 75dB, 20-s) was presented and followed by a 300-s observation period (cued fear renewal). Study members, blind to the treatments, measured freezing time during the 180-s and 300-s observation periods. The freezing time was converted to % freezing.

*2.8 Statistics*

All data are presented as mean ± standard error of the mean (SEM) and were analyzed using GraphPad Prism (GraphPad Software, version 7.0). Fear memory extinction testing and horizontal activity were analyzed by a repeated-measures two-way analysis of variance (ANOVA) using time and dose as factors. A one-way ANOVA and unpaired t-tests were used as appropriate to analyze vertical activity, total area under the fear extinction curves (AUC), and fear memory renewal. Tukey's post-hoc tests were used to compare group differences following ANOVA. The accepted level of significance was $p < 0.05$.

## 3. Results

*3.1 Immediate Ketamine*

*3.1.1 Locomotor Activity*

Horizontal activity was measured throughout the 2-h sub-anesthetic i.v. ketamine infusion administered immediately after fear conditioning. There was a significant main effect of dose $F_{(3,36)} = 11.54$ ($p < 0.001$), a non-significant effect of time $F_{(5,180)} = 1.52$ ($p = 0.18$), and a non-significant dose x time interaction $F_{(15,180)} = 1.36$ ($p = 0.17$). Post-hoc testing showed that ketamine 5 mg/kg/h significantly reduced horizontal activity at all time points as compared to saline controls (Figure 4.2A). Ketamine 1 and 10 mg/kg/h showed slightly reduced horizontal activity vs. saline, but the differences were not significant. Dose 5 mg/kg/h significantly reduced horizontal activity compared to 1 mg/kg/h at 40-min ($p < 0.01$) and between 100 - 120-min ($p < 0.05$). Dose 5 mg/kg/h also reduced horizontal activity vs. 10 mg/kg/h at 80-min ($p < 0.05$) and between 100 - 120-min ($p < 0.01$). Overall, ketamine at 5 mg/kg/h reduced horizontal activity throughout the 2-h infusion period.

Vertical (rearing) activity was measured over the final 10-min of a 2-h sub-anesthetic ketamine infusion. There were significant differences in rearing episodes across groups $F_{(3,36)}$

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= 11.2 ($p < 0.001$). Post-hoc testing showed that doses 5 and 10 mg/kg/h significantly reduced rearing episodes compared to saline (Figure 4.2C). There was no difference between dose 1 mg/kg/h and saline.
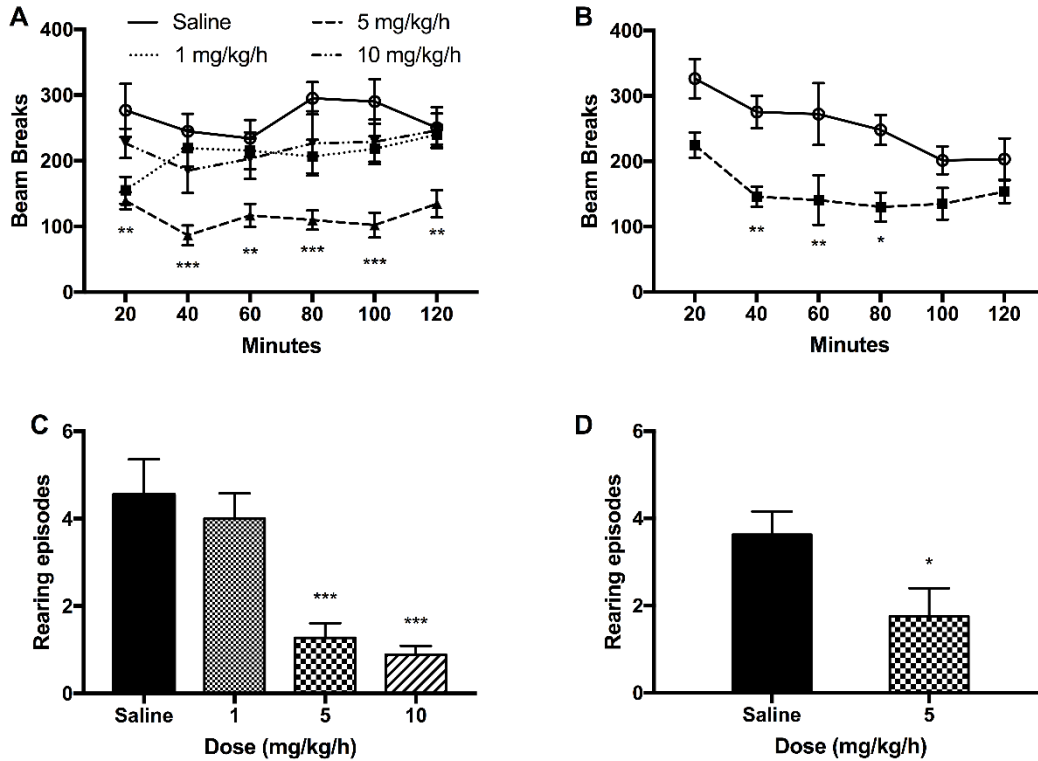


Figure 4.2. **The effect of sub-anesthetic i.v. ketamine infusion on activity.** A) A ketamine infusion (5 mg/kg/h) administered immediately after fear conditioning significantly reduced horizontal activity. B) A ketamine infusion (5 mg/kg/h) administered 1-d after fear conditioning reduced horizontal activity between 40-80-min. C) Ketamine infusions (5 and 10 mg/kg/h) administered immediately after fear conditioning reduced vertical activity (rearing episodes) observed over the final 10-min of the infusion. D) Ketamine infusion (5 mg/kg/h) administered 1-d after fear conditioning reduced rearing episodes observed over the final 10-min of the infusion. Data are shown as mean ± SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. saline)

*3.1.2 Fear Memory Retrieval and Extinction*

The effects of an immediate sub-anesthetic ketamine infusion following fear conditioning on fear memory retrieval and extinction were tested on Days 2 and 3. There were significant main effects of dose $F_{(3,36)} = 4.364$ ($p < 0.01$) and time $F_{(5, 180)} = 34.31$, but no significant dose x

time interaction $F$ (15,180) = 1.097 ($p$ = 0.36) on Day 2. Post-hoc tests showed that dose 5 mg/kg/h significantly delayed extinction vs. saline from blocks 3 through 6 (Figure 4.3A). Total area under the fear extinction curves were used to measure fear memory retrieval and there were significant differences between groups on Day 2 $F$ (3,20) = 16.84 ($p < 0.001$). Post-hoc tests showed that all ketamine infusion doses induced significant fear memory retrieval (total area) vs. saline (Figure 4.3B). Dose 5 mg/kg/h showed greater fear retrieval compared to doses 1 and 10 mg/kg/h ($p < 0.05$) indicating an inverted U relationship.

Day 3 fear retrieval and extinction testing showed a significant main effect of time $F$ (5,180) = 93.28 ($p < 0.001$), but not dose $F$ (3,36) = 2.177 ($p = 0.11$). There was a significant dose x time interaction $F$ (15,180) = 1.832 ($p < 0.05$). Post-hoc tests revealed dose 5 mg/kg/h slightly delayed extinction only at block 3 vs. saline (Figure 4.3C). There were significant differences across groups with respect to fear memory retrieval $F$ (3,20) = 8.945 ($p < 0.001$). Post-hoc tests showed that dose 5 mg/kg/h enhanced fear retrieval (total area) vs. saline on Day 3 ($p < 0.001$; Figure 4.3D). Although doses 1 and 10 mg/kg/h showed increased fear retrieval vs. saline, the differences were not significant.
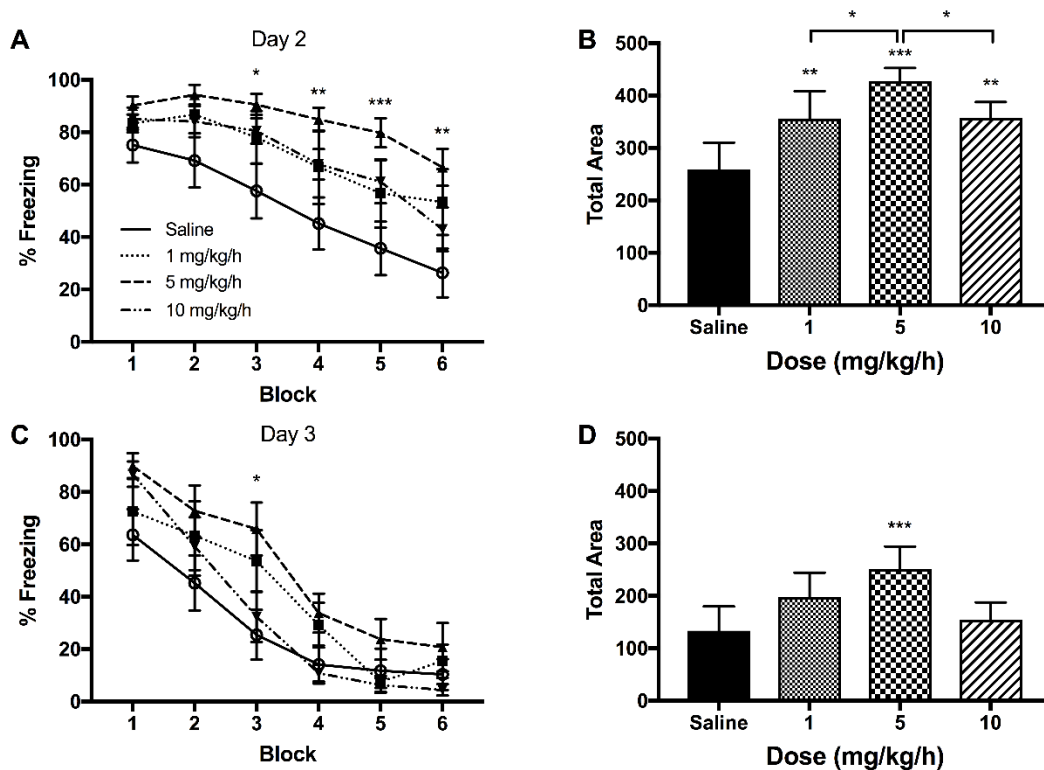
Figure 4.3. **The effect of immediate sub-anesthetic i.v. ketamine on fear retrieval and extinction.** A 2-h ketamine infusion at varying dosages or saline were administered immediately after fear conditioning on Day 0. Fear extinction was tested over 12 auditory tone trials (each block is 2 trials) in a novel context on Days 2 and 3. Fear memory retrieval was calculated using the total area under the fear extinction curves and expressed as bar graphs. A) The 5 mg/kg/h dose significantly impaired fear extinction on Day 2. B) All ketamine infusions dose-dependently enhanced fear memory retrieval (total area) on Day 2. C) Although dose 5 mg/kg/h mildly delayed fear extinction, there were no differences between groups at the conclusion of Day 3 extinction testing. D) Dose 5 mg/kg/h enhanced fear memory retrieval on Day 3. Data are shown as mean $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. saline; n = 9-11 per group).

### 3.1.3 Fear Memory Renewal

Fear memory renewal to context and cue was tested on Day 4 after rats were placed in the fear conditioning chamber (context A) and presented with a single auditory tone cue. There were significant differences in freezing responses to context $F_{(3,33)} = 7.56$ ($p < 0.001$) and cue $F$

$(3,33) = 3.19$ ($p < 0.05$). Post-hoc tests showed dose 5 mg/kg/h significantly increased freezing to the context ($p < 0.001$) and cue ($p < 0.05$) compared to saline (Figure 4.4).
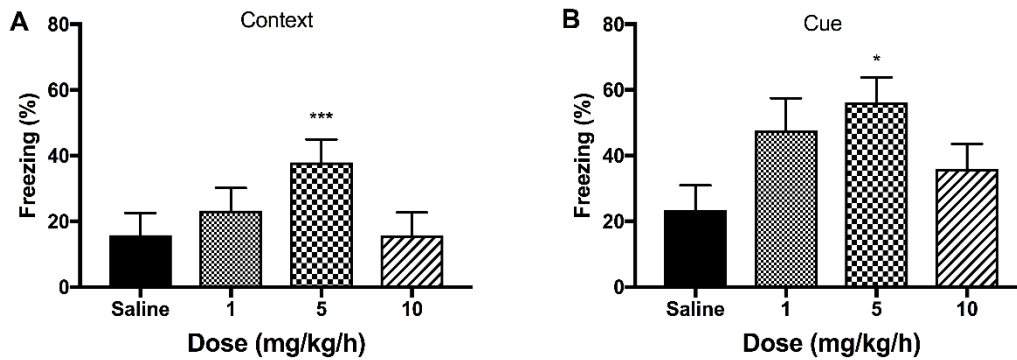


Figure 4.4. **The effect of immediate sub-anesthetic i.v. ketamine on fear memory renewal.** Rats were placed in the fear conditioning chamber (Context A) on Day 4 and underwent fear memory renewal testing to the context (freezing to context) and to a cue (freezing after 20-s auditory tone). A ketamine infusion (5 mg/kg/h) administered immediately after fear conditioning on Day 0 enhanced contextual fear renewal (A) and cued fear renewal (B) on Day 4. Data are shown as mean ± SEM (* $p < 0.05$, *** $p < 0.001$ vs. saline; n = 9-11 per group).

*3.2 Delayed Ketamine*

*3.2.1 Locomotor Activity*

Horizontal activity was measured throughout a 2-h i.v. ketamine infusion (5 mg/kg/h) administered 1-d after fear conditioning using infrared photo beams. There were significant main effects of ketamine $F(1,14) = 17.26$ ($p < 0.001$) and time $F(5,70) = 5.14$ ($p < 0.001$), but no significant ketamine x time interaction $F(5,70) = 1.025$ ($p = 0.41$). Post-hoc tests showed that ketamine dose 5 mg/kg/h significantly reduced horizontal activity compared to saline between 40 - 80-min ($p < 0.01$) (Figure 4.2B).

Vertical (rearing) activity was measured over the final 10-min of a 2-h sub-anesthetic ketamine infusion. Dose 5 mg/kg/h significantly reduced the number of rearing episodes compared to saline $t(14) = 2.236$, $p < 0.05$ (Figure 4.2D).

*3.2.2 Fear Memory Retrieval and Extinction*

The effects of a sub-anesthetic ketamine infusion administered 1-d after fear conditioning on fear memory retrieval and extinction were tested on Days 2 and 3. There were significant main effects of ketamine $F(1,14) = 11.14$ ($p < 0.01$), time $F(5,70) = 15.32$ ($p < 0.001$), and a ketamine x time interaction $F(5, 70) = 3.082$ ($p < 0.01$) on Day 2. Post-hoc tests showed that dose 5 mg/kg/h significantly delayed extinction vs. saline from blocks 5 to 6 (Fig. 4.5A). The total area under the fear extinction curves were used to measure fear memory retrieval. Ketamine dose 5 mg/kg/h increased fear retrieval (total area) vs. saline on Day 2 $t(10) = 6.4$, $p < 0.001$ (Fig. 4.5B).

Day 3 fear retrieval and extinction testing showed a significant main effect of time $F(5,70) = 41.12$ ($p < 0.001$) but not to ketamine $F(1,14) = 4.53$ ($p = 0.05$). There was no significant ketamine x time interaction $F(5,70 = 0.756$ ($p = 0.58$). Post-hoc tests showed no differences between 5 mg/kg/h vs. saline across extinction trials (Fig. 4.5C). Although there were no differences in extinction learning, dose 5 mg/kg/h displayed increased fear retrieval vs. saline on Day 3 $t(10) = 4.574$, $p < 0.01$ (Fig. 4.5D).
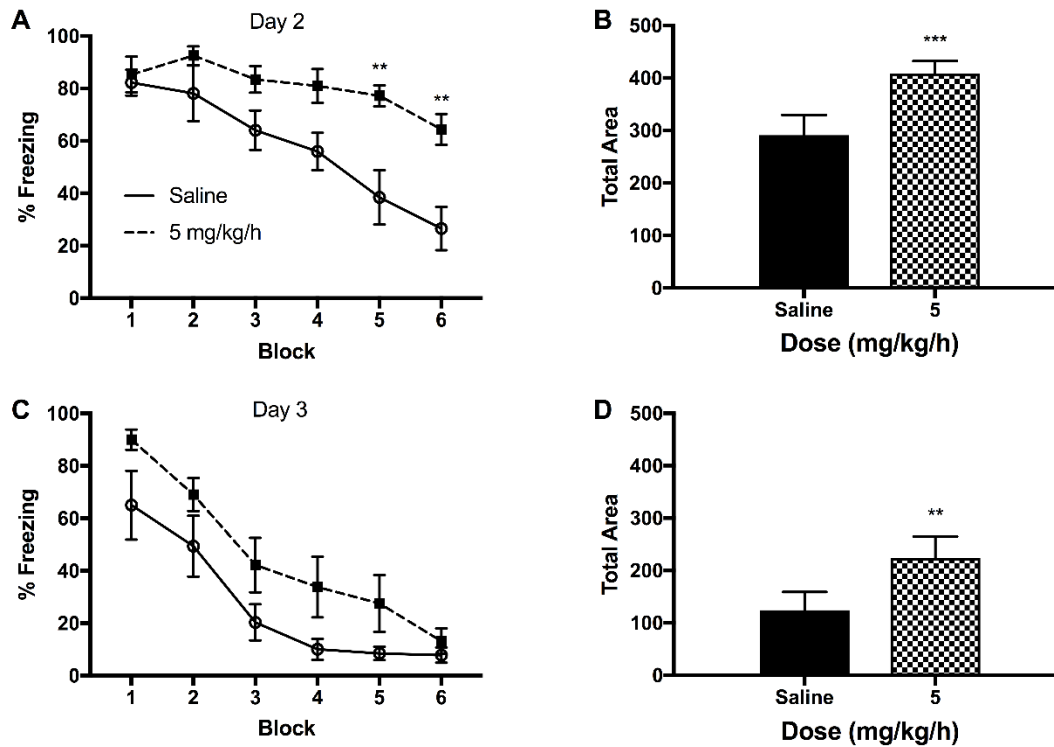
Figure 4.5. **The effect of delayed sub-anesthetic i.v. ketamine on fear-extinction.** A 2-h ketamine infusion (5 mg/kg/h) or saline were administered 1-d after fear conditioning on Day 1. Fear extinction was tested over 12 auditory tone trials (each block is 2 trials) in a novel context on Days 2 and 3. Fear memory retrieval was calculated using the total area under the fear extinction curves and expressed as bar graphs. A) The ketamine infusion significantly impaired fear extinction and (B) enhanced fear memory retrieval (total area) on Day 2. C) There were no differences in extinction between groups on Day 3 although (D) the ketamine infusion group showed enhanced fear memory retrieval. Data are shown as mean ± SEM (** $p < 0.01$, *** $p < 0.001$ vs. saline; n = 8 per group).

*3.2.3 Fear Memory Renewal*

Fear memory renewal to context and cue were tested on Day 4 after rats were placed in the fear conditioning chamber (context A) and presented with a single auditory tone cue. Ketamine dose 5 mg/kg/h significantly increased freezing to context $t(14) = 2.912$, $p = 0.01$ (Fig. 4.6A). The ketamine group showed increased freezing to cue vs. saline but the difference was not significant $t(14) = 1.821$, $p = 0.09$ (Fig. 4.6B).
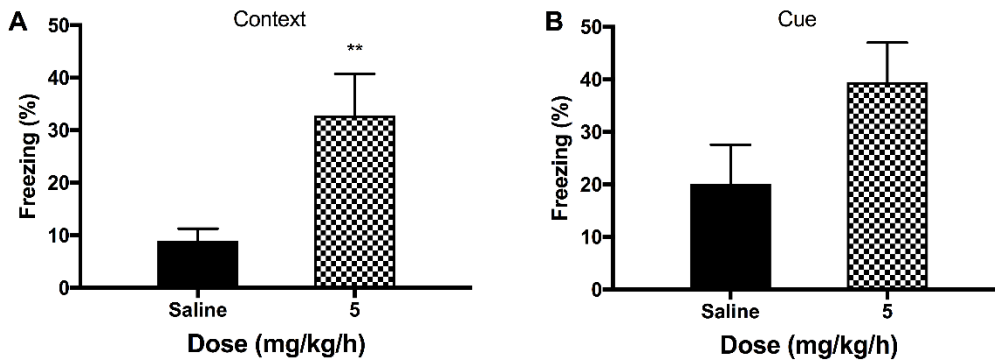
Figure 4.6. **The effect of delayed sub-anesthetic i.v. ketamine on fear memory renewal.** Rats were placed in the fear conditioning chamber (Context A) on Day 4 and underwent fear memory recall testing to the context (freezing to context) and to a cue (freezing after 20-s auditory tone). A ketamine infusion (5 mg/kg/h) administered 1-d after fear conditioning on Day 1 enhanced renewal to context (A) but not to cue (B) on Day 4. Data are shown as mean ± SEM (** $p < 0.01$; n = 8 per group).

## 4. Discussion

Sub-anesthetic i.v. ketamine is often administered to provide sedation and analgesia to the traumatically wounded. Although a seemingly ideal trauma anesthetic due to its cardio-pulmonary stability and high-safety ceiling [6], ketamine also induces transient psychoses such as hallucinations, delusions, and dissociation [9, 129] that may exacerbate trauma and stress related disorders. While ketamine is administered i.v. to humans, most pre-clinical rodent models utilize i.p. injections that do not maintain an elevated drug plasma concentration over time. Therefore, we administered a 2-h sub-anesthetic i.v. ketamine infusion to rodents either immediately or 1-d after fear conditioning and observed reduced activity, enhanced fear memory retrieval, delayed fear extinction, and enhanced fear memory renewal.

Sub-anesthetic ketamine infusions administered either immediately or delayed after fear conditioning, dose-dependently reduced locomotor activity. We measured vertical (rearing) and horizontal activity because it allowed us to verify the dose-dependent ketamine infusion effects on behavior. Interestingly, the ketamine 5 mg/kg/h infusion reduced horizontal activity compared

to saline and ketamine doses at 1 and 10 mg/kg/h. The reduced horizontal activity observed at 5 mg/kg/h replicates our previous findings [230] and are in line with others that also observed reduced locomotor activity following low-dose ketamine i.p. injections [154-156]. In comparison, ketamine at higher doses induce hyper- not hypo-activity [148, 149]. The suppressed locomotor effects of lower dose ketamine are unknown but may result from mild sedative effects while sparing motor activity stimulation.

In addition to reduced horizontal activity, we observed suppressed rearing activity among both the 5 and 10 mg/kg/h treatment groups. These results are in agreement with previous behavioral findings from our laboratory and others at sub-anesthetic ketamine doses [155, 230]. The reduced rearing behavior observed at 10 mg/kg/h most likely resulted from an unstable gait and mild ataxia observed at this moderate dosage. Moreover, reduced rearing at 10 mg/kg/h was not due to hyper-locomotive effects because the horizontal activity of these rats was similar to the 1 mg/kg/h and saline groups that showed normal rearing behavior. In contrast, rats that received 5 mg/kg/h displayed a steady gait and suppressed rearing behavior. These rats often appeared hypo-locomotive and backed into a corner of the infusion chamber. Typically, rodent rearing behavior in a novel context demonstrates increased exploratory behavior, attentiveness, and a reduced perceived threat [248]. Taken together, the suppressed horizontal and rearing activity observed at 5 mg/kg/h may indicate either a sedated (non-dissociative) state induced by an analgesic ketamine response or possibly a state of increased perceived threat.

Sub-anesthetic ketamine infusions administered either immediately or 1-d after fear conditioning enhanced fear memory retrieval and delayed fear extinction. The increased total area under the extinction curves on Days 2 and 3 indicated an enhanced fear memory retrieval. A robust fear memory consolidation leads to increased fear memory retrieval, however, previous literature that describes ketamine effects on memory consolidation are mixed. Investigators

reported that ketamine i.p. injections at both sub-anesthetic and anesthetic doses either impair [249, 250], show no effect [136], or enhance memory [10, 251]. To further explore a consolidation effect, we administered a ketamine infusion 1-d after fear conditioning and observed increased fear memory retrieval over two extinction testing days, similar to the immediate ketamine infusion experiment. These results suggest that the fear memory enhancing effects of ketamine may be independent of a fear memory consolidation process. In accordance with our findings, others also reported enhanced fear memory retrieval after sub-anesthetic and anesthetic ketamine i.p. injections [138, 189].

A sub-anesthetic ketamine infusion (5 mg/kg/h) administered immediately or 1-d after fear conditioning delayed fear extinction. Our results are in agreement with others that observed MK-801 and phencyclidine, NMDA receptor antagonists similar to ketamine, to also impair fear extinction [143, 144, 252]. Additionally, a partial NMDA receptor agonist, d-cycloserine, enhances fear extinction [253], which further supports the opposing effects of glutamatergic signaling on fear extinction learning. However, our results conflict with a recent investigation that reported enhanced fear extinction among rats that received a single ketamine i.p. injection (10 mg/kg) 1-d after fear conditioning [140]. These investigators observed enhanced fear extinction on the second of three-extinction testing days suggesting a delayed, time-specific effect. In contrast, McGowan et al. administered a single ketamine i.p. injection (30 mg/kg) to mice either 1h or 1-week after fear conditioning and found no effect on fear extinction [254]. A key difference between the previous studies and the present investigation is the route of ketamine administration (i.v. infusion vs. i.p. injection). It is conceivable, that the infusion route we utilized maintained a prolonged and elevated ketamine concentration in the rodent brain, which negatively impacted neuronal circuitry required for extinction learning. Brain structures such as the mPFC and the hippocampus are known to undergo alterations after NMDA receptor

antagonism [238, 255-261]. If these brain structures are negatively impacted by a sustained ketamine infusion, it could result in an impaired top-down regulatory control of amygdala dependent freezing behavior and delay extinction learning.

A potential mechanism of neuronal alteration induced by ketamine is paradoxical hyper-glutamate activity. NMDA receptor blockade on pre-synaptic inhibitory GABAergic neurons leads to increased pre-synaptic glutamate release [238]. Glutamate initiates a post-synaptic signaling cascade that activates the mammalian target of rapamycin (mTOR) pathway and produces mediators associated with the therapeutic effects of ketamine such as anti-depressant-like effects [119, 140]. On the other hand, extended pre-synaptic NMDA blockade of GABAergic neurons can lead to glutamatergic dysregulation and contribute to a range of neuropsychiatric disorders to include PTSD and schizophrenia [239, 240]. Excessive glutamatergic signaling can induce neuronal toxicity and apoptosis through a dysfunctional influx of calcium and sodium ions, especially in the prefrontal cortex [239]. Opposing effects of glutamate (therapeutic vs. detrimental) are possibly related to the duration of ketamine exposure. The one time, low-dose ketamine i.p. injection utilized in rodent depression models appears to enhance synaptic function, while a continuous sub-anesthetic ketamine i.v. infusion may dysregulate glutamatergic signaling and negatively alter neuronal structures.

If extinction learning is impaired, then it is possible to observe enhanced fear renewal, which is a type of fear extinction reversal. In fact, we observed a time-dependent ketamine effect on fear renewal following ketamine infusions. Immediate ketamine infusions enhanced fear renewal to context and cue, while the delayed infusion enhanced renewal only to context and not cue. Our fear renewal results disagree with a recent investigation that reported decreased fear renewal in response to an auditory cue in the fear conditioning context measured 7-d after fear extinction [140]. Our differences could be related to the total number of extinction trials. Girgenti et al.

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performed 36 extinction trials over 3 days compared to 24 trials over 2 days in our design. More extinction trials may have produced greater extinction learning; however, our ketamine treated rats displayed a similar extinction pattern as our saline control group suggesting our extinction method was adequate. Additionally, Girgenti et al. tested renewal 7-d after extinction whereas we tested renewal 1-d after extinction. It is possible that testing renewal shortly after extinction learning versus waiting a longer period could contribute to enhanced renewal [262], suggesting a time-dependent renewal effect. Interestingly, only the immediate, not the delayed, ketamine infusion enhanced renewal to the auditory cue. This implies that an immediate post-fear conditioning ketamine infusion may strengthen fear memory associations between the conditioned and unconditioned stimuli during amygdala consolidation whereas, the delayed infusion had no such affect because it was administered after the initial encoding.

Finally, we observed a dose-dependent ketamine effect on fear memory retrieval, extinction, and renewal that represented an inverted U response. Ketamine dose 5 mg/kg/h produced increased freezing behavior, while doses at 1 and 10 mg/kg/h had reduced effects. These results are in accordance with others that reported a similar inverted U dose-response between corticosterone and fear behaviors [212, 213]. Low and high doses of CORT had no effect, while intermediate CORT dosing enhanced fear memory consolidation and conditioned freezing. Interestingly, i.v. ketamine infusions dose-dependently elevate CORT, a known mediator of fear memory [263]. Therefore, the inverted U dose-response of ketamine fear memory may be related to ketamine induced CORT expression, where low and high CORT levels impair memory and while intermediate CORT levels enhance memory.

This investigation has several limitations. First, we utilized a compressed timeline to assess fear memory retrieval, extinction, and renewal. Our findings may indicate short-term ketamine effects and therefore, interpreting these results through a PTSD construct may be problematic.

Instead, these findings may align closer to an acute stress disorder construct, which resemble PTSD-like symptoms within the first month after a trauma. Additionally, we cannot rule out a short term pharmacologic effect of ketamine due to the compressed experimental timeline. Assessing fear extinction and renewal at extended time points (i.e. 10-30 days later) would better evaluate any protracted effects of ketamine on fear behaviors. Secondly, we did not co-administer a benzodiazepine with ketamine, which is used to suppress adverse dissociative effects in clinical practice. We anticipated that a co-administrated psychoactive agent with GABA-ergic properties might confound ketamine effects. Lastly, we used only male rats. Previous work has shown anesthetic and sub-anesthetic ketamine to induce sex-related differences on metabolism and anti-depressant-like effects in rodents [199-201]. There are also sex-related differences in response to stress and anxiety, therefore, we cannot generalize our results to female rats.

## 5. Conclusion

First responders and clinicians administer ketamine to injured trauma victims to provide rapid sedation and analgesia. Since ketamine is known to induce transient psychosis and dissociation, there are concerns regarding potential influences on trauma and stress related disorders. We administered a 2-h sub-anesthetic i.v. ketamine infusion at varying doses either immediately or 1-d after fear conditioning. Our findings show that a ketamine infusion (5 mg/kg/h) suppressed activity, increased fear memory retrieval, delayed fear extinction, and enhanced fear renewal suggesting a detrimental effect of post-trauma ketamine. To our knowledge, these are novel findings in that we are the first to administer a continuous sub-anesthetic i.v. ketamine infusion after fear conditioning to evaluate fear related behaviors in male rats.

**Disclaimer:** The views expressed in this article are those of the authors and do not reflect official policy or position of the Uniformed Services University of the Health Sciences, Department of the Navy, the Department of Defense, or the United States Government.

# CHAPTER 5

## 1. Conclusions

The first objective of this proposal was to determine a range of effective sub-anesthetic i.v. ketamine dosages in rats. Ketamine is most commonly administered to humans via the i.v. route either as a bolus and/or an infusion; however, most pre-clinical investigations administer ketamine to rodents via i.p. injection. Significant differences between the two delivery routes with respect to pharmacokinetics and pharmacodynamics can hinder translation to human investigations. There were no published reports, to our knowledge, that described sub-anesthetic i.v. ketamine infusion dosages and effects on rodent behavioral measures. Therefore, we administered two bolus ketamine doses (2 and 5 mg/kg) and three i.v. infusion dosages (5, 10, and 20 mg/kg/h) and measured effects on locomotion, nociception, dissociation, and sensorimotor gating in male Sprague-Dawley rats.

Our ketamine dose-response study provided necessary information that informed our future investigations. Most notably, our larger ketamine dosages (5 mg/kg bolus and 20 mg/kg/h infusion) impaired pre-pulse inhibition (PPI), which is a measure of sensorimotor gating. Sensorimotor gating impairments are not only observed among schizophrenia patients but also among those diagnosed with PTSD [264-267]. These results provided an indication that a potential relationship between ketamine and stress related disorders may exist. We also observed increased ketamine dosages to induce long-lasting antinociception with significant dissociative behaviors. These increased dosages may translate to the larger doses that a combat medic may utilize to induce sedation and intense analgesia in the field. Interestingly, we observed a lower ketamine infusion dose (5 mg/kg/h) to suppress both vertical (rearing) and horizontal locomotor activity with less dissociative behaviors. The behaviors observed at the lower ketamine infusion

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dosage resembled the mild dissociative effects reported by patients receiving therapeutic low-dose ketamine infusions for treatment refractory MDD and PTSD [115, 117]. The range of dose-dependent behaviors we observed in this dose-finding study directed us to utilize a similar range of ketamine dosages (low to moderate) for our remaining investigations. Overall, this initial dose-response study validated an i.v. ketamine infusion model in rats and provided novel information regarding sub-anesthetic i.v. ketamine dosages.

Our second objective was to characterize the dose-dependent effects of sub-anesthetic i.v. ketamine infusions on CORT and BDNF following fear conditioning. CORT, a stress hormone, and BDNF, a neurotrophin associated with synaptic plasticity, are both implicated as mediators of memory formation. Although ketamine effects on CORT are well-described among humans, there are limited pre-clinical investigations exploring effects of i.p. ketamine injections on CORT levels and none that utilize i.v. ketamine infusions among rodents. Additionally, there is an absence of pre-clinical work that explores sub-anesthetic i.v. ketamine infusion effects on BDNF. Therefore, we administered 2-h sub-anesthetic i.v. ketamine infusions (0, 5, or 20 mg/kg/h) to male Sprague-Dawley rats following exposure to a fear conditioning paradigm and measured post-infusion plasma CORT immediately and plasma BDNF 2-h later.

We observed a significant dose-dependent increase in the CORT response immediately after the ketamine infusion. There are mixed results among pre-clinical studies regarding the effects of CORT elevations on fear memory consolidation [69, 235] and none that describe a sustained elevation of CORT induced by a continuous i.v. ketamine infusion. Interestingly, others have described an inverted U dose response of CORT on fear memory consolidation, where an intermediate dose enhanced memory while low and high doses had no effect [213, 268]. Although we did not measure behavioral outcomes in this investigation, our dose-

dependent ketamine effects on CORT expression are interesting and suggest a possible CORT linkage between post-trauma ketamine administration and fear memory consolidation.

We observed a significant plasma BDNF reduction after a prolonged high-dose ketamine infusion (20 mg/kg/h). The ketamine effects on BDNF are mixed in both human and rodent investigations. A low-dose i.p. ketamine injection (10 mg/kg, i.p.) increases BDNF in rodent depression models [119]; however, blood BDNF is suppressed among chronic ketamine abusers and brain BDNF is reduced among rats that receive sub-anesthetic ketamine injections (25 mg/kg, i.p.) over 1-week [227, 228]. Therefore, it appears that ketamine effects on BDNF may be dose and time dependent. Prolonged ketamine exposures at increased dosages as seen in chronic administration paradigms, similar to our infusion model, appear detrimental towards BDNF production. In contrast, a low-dose, single ketamine injection improves BDNF levels. Overall, our BDNF results are difficult to interpret as we did not measure behavioral outcomes. The CORT and BDNF biomarker results from this investigation were necessary first steps towards the completion of our third objective.

Our third objective was to measure the effect of an immediate or delayed sub-anesthetic i.v. ketamine infusion on fear memory behaviors. Ketamine is a multimodal dissociative anesthetic and potent analgesic administered in the immediate post-trauma period to injured victims; however, transient psychosis effects such as hallucination, delusions, agitation, and dissociation have raised concerns regarding ketamine's effect on adverse psychological impacts surrounding trauma. There are a limited number of post-trauma ketamine studies in humans, which have produced mixed results [130-133]. Similarly, there are a limited number of post-fear conditioning ketamine investigations in rodents that have also produced mixed results [136-141, 180]. There are no rodent fear conditioning studies that have utilized the clinically relevant i.v. ketamine infusion model. Therefore, we administered 2-h sub-anesthetic i.v. ketamine infusions

(0, 1, 5, or 10 mg/kg/h) immediately or 1-day after (5 mg/kg/h) after footshock fear conditioning and measured effects on fear memory retrieval, fear extinction, and fear renewal.

Immediate (1, 5, and 10 mg/kg/h) and delayed (5 mg/kg/h) ketamine infusions after fear conditioning enhanced fear memory retrieval. Our observed increase in fear memory retrieval is in line with another investigation that administered post-fear conditioning ketamine injections to rats and reported increased fear memory 1-month later [137]. We conducted a second experiment to examine a possible consolidation effect and administered a ketamine infusion (5 mg/kg/h) 1-day after fear conditioning and found similar enhanced fear memory retrieval effects. Our delayed ketamine results are in agreement with a recent investigation that administered a single ketamine injection to rats 6-days after fear conditioning and reported increased freezing behavior the next day in response to a situational reminder [139].

Immediate and delayed ketamine infusions after fear conditioning impaired fear extinction learning. Our results disagree with others that report post-fear conditioning ketamine to either facilitate [140] or have no impact [141] on fear extinction. We also observed increased fear renewal among rats exposed to post-fear conditioning ketamine infusions. Our results, conflict with a recent investigation that reported reduced fear renewal among rats that received a post-fear conditioning ketamine injection [140]. Interestingly, we observed different fear renewal effects according to infusion timing. The immediate post-fear conditioning ketamine infusion enhanced renewal to both context and cue, while the delayed ketamine infusion enhanced renewal only to context. However, differences in fear conditioning, number of extinction trials, rodent species, and ketamine dose, timing, and route of administration may factor into differing results across studies.

The mechanisms of enhanced fear-memory effects of post-fear conditioning ketamine are largely unknown; however, I have proposed three hypotheses that may explain our observed phenomenon.

*CORT hypothesis*

It is understood that CORT released in the immediate period after a stress-provoking event acts to enhance memory consolidation. It follows that remembering a fearful or stressful event is an evolutionary protective mechanism. The memory enhancing effects of CORT have been well-studied and validated in both pre-clinical and clinical investigations [213, 269-271] and it appears that CORT also requires concurrent nor-adrenergic stimulation to further enhance memory [212]. Interestingly, CORT effects on memory appear as an inverted U dose response in that low and high doses show less effects, while intermediate CORT doses show great memory enhancement. We observed sub-anesthetic i.v. ketamine infusions to dose-dependently increase CORT levels in rats (Fig. 3.2). Also, ketamine is a sympathomimetic agent that increases heart rate and blood pressure. Taken together, an i.v. ketamine infusion administered after fear-conditioning leads to dose-dependent elevations in CORT along with an adrenergic response that may contribute to our inverted U-dose enhanced fear memory in this investigation.

*Retrograde Facilitation hypothesis*

A phenomenon of enhanced memory consolidation is observed in alcohol consumption studies [272, 273]. These studies describe enhanced memory of tasks that occur prior to alcohol consumption. A proposed mechanism is that alcohol induces anterograde impairment through disruption of new information after alcohol exposure. Disruption of post-alcohol information, in effect, reduces information interference and allows regions such as the hippocampus, to devote memory resources to events that occurred prior to alcohol exposure, which is termed retrograde facilitation. Retrograde facilitation effects have been observed with midazolam [274] and

propofol [275, 276]. A similar process may occur with post-fear conditioning ketamine in that ketamine disrupts the encoding of new information presented post-infusion (anterograde impairment), which in turn, allows limbic systems to focus memory resources on the fear conditioning event that occurred pre-infusion (retrograde facilitation).

*Hyper-glutamate hypothesis*

The increased CORT and retrograde facilitation hypotheses are plausible mechanisms towards enhanced memory consolidation but are inadequate in their explanation of our observed ketamine impairment of fear extinction. Instead, a possible mechanism is related to an induced hyper-glutamatergic response secondary to the continuous i.v. ketamine infusion. Ketamine is known to antagonize pre-synaptic NMDA receptors on GABAergic neurons [238]. Disinhibited GABAergic control results in a paradoxical pre-synaptic glutamate release [238]. A sustained, hyper-glutamatergic response induced by a continuous i.v. ketamine infusion may lead to excitotoxicity of key regions that facilitate fear extinction learning, such as the mPFC [277, 278]. Therefore, impaired top-down regulatory control of amygdala driven fear responses may lead to disrupted fear extinction.

Taken together, a combination of these three mechanism may best explain the enhanced memory consolidation and impaired fear extinction induced by a continuous sub-anesthetic but analgesic i.v. ketamine infusion. A ketamine infusion may enhance hippocampal (context) and amygdala (context and cue association) memory consolidation, while inducing excitotoxicity in the mPFC and impairing subsequent fear extinction learning.

In summary, our utilization of an i.v. ketamine infusion proved a novel and clinically relevant drug delivery model for rodents. Ketamine infusions induce dose-dependent dissociative behaviors, antinociception, altered locomotion, and sensorimotor gating deficits. Continuous i.v. ketamine infusions also induce prolonged, dose-dependent elevations in CORT and reductions in

plasma BDNF, which are known mediators of fear memory. Lastly, there is a dose-dependent relationship between sub-anesthetic i.v. ketamine infusions administered post-fear conditioning on enhanced fear memory retrieval, delayed fear extinction, and enhanced fear memory renewal. These studies indicate that a continuous sub-anesthetic but analgesic i.v. ketamine infusion after traumatic event exposure is detrimental to the development of trauma and stressor related disorders.

## 2. Future Research

The investigations we conducted throughout this dissertation proposal have spawned further questions and several lines of new research interests. First, we utilized a compressed timeline (5-days) to study post-fear conditioning ketamine infusion effects on fear memory retrieval, extinction, and renewal. The behavioral outcomes we measured may in fact, more accurately model acute stress disorder, which are PTSD symptoms that manifest in the first 30-days after a traumatic event. For a future study design, we would propose a longer time interval (i.e. 10-days or more) between the post-fear conditioning ketamine infusion and fear behavior measurement. A longer interval between treatment intervention and outcome measurement would extend beyond protracted pharmacologic effects of ketamine and more closely translate to a time-frame used to make a clinical PTSD diagnosis.

Next, we are currently in the early stages of a proposal development to study post-fear conditioning ketamine effects on fear memory behaviors among female rats. The NIH has called for the inclusion of sex and gender across biomedical research due to the overwhelming neglect of female subjects in most pre-clinical investigations [279]. Moreover, the DoD recently made policy changes to fully open all military occupations, including combat roles, to women [280]. These policy changes mean that combat medics and military medical providers could provide

treatment to larger numbers of combat wounded female service members in future military conflicts. Therefore, understanding sex-related differences related to post-trauma anesthetics, such as ketamine, on fear memory and stress related disorders is of significant interest to military commanders, military health leaders, and policy stakeholders.

Lastly, we aim to expand our research interests beyond ketamine to a recently discovered active ketamine metabolite along with other intravenous anesthetics. A recent investigation has reported hydroxynorketamine (HNK), an active ketamine metabolite, to mediate the antidepressant effects of ketamine [205]. HNK reverses depressive-like behavior in mice while sparing the dissociative, anesthetic, and addictive properties of the parent compound, ketamine. Thus, we are interested in possible HNK mediated effects towards PTSD-like behaviors either as a treatment or potential preventative agent after trauma exposure. Expanding beyond ketamine, we are also interested in exploring other i.v. anesthetics, such as dexmedetomidine. Dexmedetomidine is an alpha-2 agonist that provides sedation and analgesia while sparing respiratory depression. A recent investigation reported that a single dexmedetomidine injection to rats after inescapable footshock reduced subsequent freezing to a situational reminder suggesting a possible preventative effect [138]. There is limited research exploring relationships between dexmedetomidine and PTSD and thus, we aim to expand this line of work in the future.

## 3. Clinical Significance

Ketamine is a multimodal dissociative anesthetic and potent analgesic administered to the combat wounded following traumatic injury. Cardiopulmonary stability and a large safety window has led the U.S. DoD committee on Tactical Combat Casualty Care (TCCC) to endorse ketamine as a battlefield analgesic that could supplant morphine in the future. Ketamine, however, induces transient psychosis effects such as hallucination, delusions, agitation, and

dissociation that may impact the development of stress related disorders if administered in the peri-trauma period. Thus, there are clinical questions aimed at the effects of post-trauma ketamine administration on the development of stress related disorders. Our pre-clinical investigations attempted to answer these questions by using a clinically relevant sub-anesthetic i.v. ketamine infusion in a rodent model of PTSD.

Our results suggest that post-trauma ketamine exposure adversely impacts fear memory and fear extinction. We understand these results are somewhat controversial as ketamine has recently gained significant media attention as an off-label treatment for MDD and PTSD, while also receiving endorsements as a safe battlefield analgesic. Supporters of post-trauma ketamine often cite the work of McGhee et al., which found no relationship between intraoperative ketamine administration and PTSD diagnosis among U.S. service members treated for combat related burn injuries [131]. However, McGhee et al. did not report the time interval between point of injury and intraoperative ketamine exposure along with the co-administration of other psychoactive anesthetics that could impact PTSD development. In contrast, Schonenberg et al. administered post-trauma ketamine to victims of moderate traumatic injury and reported a significant increase in ASD and dissociation symptoms compared to opioid and non-opioid treatment groups [133]. Similarly, pre-clinical researchers have also reported adverse PTSD-like behavior outcomes after ketamine treatment in recent investigations [137-139].
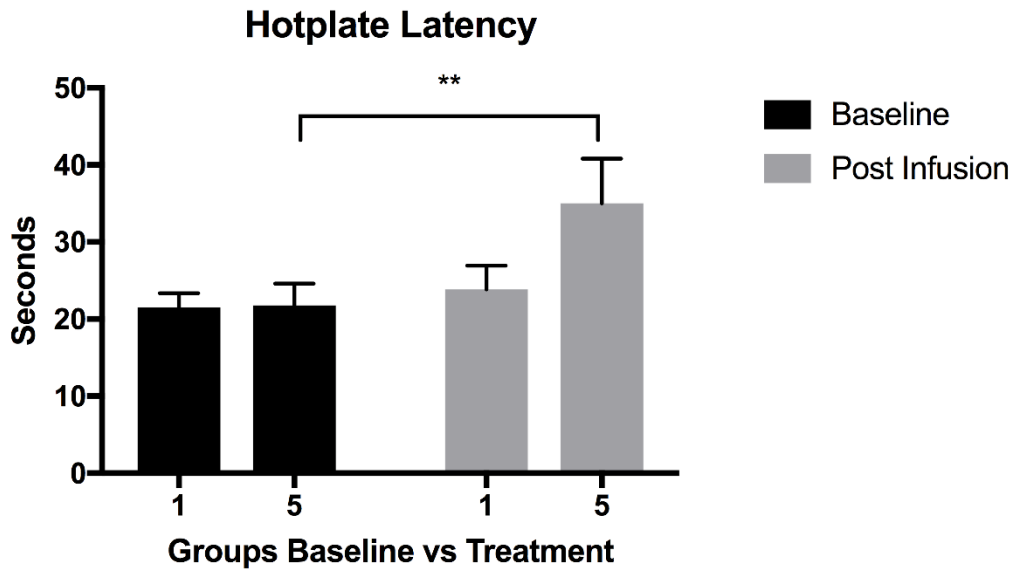
## Hotplate Latency



Figure 5.1 **The effect of 2-h ketamine infusions on thermal nociception.** Rats underwent 2-h sub-anesthetic ketamine infusions (1 or 5 mg/kg/h) and were placed on a hotplate immediately post-infusion. Latency was measured in seconds from hotplate placement to lift and lick of rear hind paw. A ketamine infusion at 5 mg/kg/h produced significant antinociception compared to baseline. Data are shown as mean $\pm$ SEM (** $p < 0.01$; n = 8 per group).

It is difficult to recommend a change in clinical practice at this time based upon our results. Interestingly, we observed an inverted U dose response in that the highest and lowest ketamine infusion doses did not adversely impact fear extinction and fear renewal, while the intermediate dose was detrimental. Since our lowest ketamine infusion dose (1 mg/kg/h) did not produce post-infusion antinociception (Figure 5.1), we would suggest that clinicians error on the side of higher ketamine doses during post-trauma care. The higher ketamine dosage range (10 mg/kg/h) seemed to have the least impact on rodent fear behaviors. Additionally, benzodiazepines are commonly co-administered with ketamine in clinical anesthesia practice to suppress dissociative effects. Benzodiazepines are not co-administered with ketamine for battlefield analgesia. We did not co-administer a benzodiazepine in our fear memory research because we anticipated confounding results with the addition of another psychoactive

medication. Since benzodiazepines are GABA agonists, they may function to offset the proposed hyper-glutamatergic response of ketamine and offer protective effects towards ketamine induced fear memory impairments. Therefore, we recommend that clinicians consider co-administering benzodiazepines with ketamine until future investigations can examine this relationship with respect to PTSD. In summary, we recognize that medical care following traumatic injury is highly complex and involves the administration of multiple pharmacologic agents that may impact the neurobiology of stress related disorders; however, we anticipate that our pre-clinical sub-anesthetic i.v. ketamine findings will add to the body of knowledge and assist clinicians as they determine best practice standards, while informing future clinical investigations.

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