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Dissecting Comorbidity Between Opioid Use/Dependence and Post-Traumatic Stress Disorder

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Dissecting Comorbidity Between Opioid Use/Dependence and Post-Traumatic Stress Disorder

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Psychology

by

Zachary Thomas Pennington

2018

ABSTRACT OF THE DISSERTATION

Dissecting Comorbidity Between Opioid Use/Dependence and Post-Traumatic Stress Disorder

by

Zachary Thomas Pennington

Doctor of Philosophy in Psychology

University of California, Los Angeles, 2018

Professor Michael S. Fanselow, Chair

The focus of this dissertation is to explore how opioid use and dependence might promote the development and persistence post-traumatic stress disorder (PTSD). In addition to giving further insight into the etiology of PTSD, this research sheds important light on the ramifications of opioid use, whose licit and illicit use has skyrocketed in recent years. The contained experiments explore the relationship between opioid use and PTSD from a behavioral-mechanistic stance, addressing the precise cognitive processes related to fear impacted by opioid use, and moreover, begin to address the receptor systems through which opioid use potentiates fear learning. The experiments presented also begin to examine how trauma influences associative learning about drugs of abuse in order to understand one of the largest predictors of substance use disorders: trauma.

Utilizing a rodent model of PTSD in conjunction with various pharmacological manipulations, it is demonstrated that chronic exposure to opioids is able to produce a lasting enhancement in the mechanisms that support fear learning. This enhancement was found to be

independent of changes in pain sensitivity and anxiety, and was also shown not be a consequence of the stress of repeated withdrawal. Moreover, opioid-induced changes in fear learning were accompanied by markers of increased plasticity within the basolateral amygdala, a critical site for associative fear learning. These findings have serious implications for the medicinal and non-medicinal use of opioids, particularly given high rates of opioid prescriptions amongst those who undergo physical traumas.

With respect to receptor systems, although dynorphin release and subsequent kappa receptor activation has been proposed to support increased anxiety as a consequence of drug use, kappa antagonism failed to counter opioid-induced increases in fear learning. Moreover, counter to recent research suggesting anxiolytic properties of kappa antagonism, we were unable to detect any anxiolytic efficacy of a kappa receptor antagonist. It is likely that the conditions under which dynorphin release supports anxiety are limitied, and their therapeutic efficacy must be considered in this light.

Lastly, prior traumatic experience was found to robustly increase opioid sensitivity. However, counter to the initial hypothesis, trauma was unable to augment associative learning about opioids in the conditioned place preference task. This finding suggests that the potentiation of amygdala-dependent learning by trauma is restricted to aversive associations. Future research hopes to further explorer the relevance of heightened opioid sensitivity to drug-seeking behaviors.

The dissertation of Zachary Thomas Pennington is approved.

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2018

DEDICATION

In memory of Emanuele Seu. Eman, thank you for being such an extraordinary mentor and friend.

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LIST OF ABBREVIATIONS

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

APV - (2*R*)-amino-5-phosphonovaleric acid; (2*R*)-amino-5-phosphonopentanoate; NMDA antagonist

BLA – Basolateral amygdala

BNST – Bed nucleus of the stria terminalis

CEA – Central amygdala

CPP – Conditioned place preference

EPM – Elevated plus maze

GWAS – Genome wide association study

mPFC – Medial prefrontal cortex

mRNA – Messenger ribonucleic acid

NMDA - N-methyl-D-aspartate

PTSD – Post-traumatic stress disorder

SEFL – Stress enhanced fear learning

SUD – Substance use disorder

vmPFC – Ventromedial prefrontal cortex

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Introduction

The majority of individuals will experience a life-threatening and incredibly stressful event at one point in their lives, be it sexual assault, childhood abuse, military trauma, or living through a natural disaster (Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995; Kilpatrick et al., 2013). For a subset of these individuals, representing approximately 8% of the total United States population, such trauma will produce the persistent and debilitating symptoms of post-traumatic stress disorder (PTSD) (Kessler et al., 1995; Kilpatrick et al., 2013). PTSD symptoms include intrusive and distressing memories of the traumatic event, nightmares and sleep difficulty, generalized anxiety, difficulty concentrating on daily tasks, and avoidance of important life activities (American Psychiatric Association, 2013). The burden of PTSD on the individual and society are large, with a staggering number of individuals with PTSD finding employment difficult, developing other psychiatric conditions and chronic health issues, and being at an increased risk for attempting suicide (Kessler, 2000).

Although affective cognitive-behavioral therapies and pharmacotherapies exist for PTSD, symptoms fail to abate in a substantial proportion of affected individuals (Bradley, Greene, Russ, Dutra, & Westen, 2005; Cukor, Olden, Lee, & Difede, 2010), necessitating the discovery of novel therapeutic approaches. Moreover, given the number of military personnel and emergency responders at high risk for experiencing trauma, the ability to either build resilience in at-risk populations or select individuals based upon factors conferring resilience, is needed. A more thorough understanding of the vulnerability factors for PTSD and the biological changes that support symptom development holds promise for illuminating a path for novel intervention/prevention strategies.

The focus of this dissertation is to explore how opioid use and dependence might promote the development and persistence post-traumatic stress disorder (PTSD). In addition to

giving further insight into the etiology of PTSD, this research sheds important light on the ramifications of opioid use, whose licit and illicit use has skyrocketed in recent years. The contained experiments explore the relationship between opioid use and PTSD from a behavioral-mechanistic stance, addressing the precise cognitive processes related to fear impacted by opioid use, and moreover, begin to address the receptor systems through which opioid use potentiates fear learning. The experiments presented also begin to examine how trauma influences associative learning about drugs of abuse in order to understand one of the largest predictors of substance use disorders: trauma.

Utilizing a rodent model of PTSD in conjunction with various pharmacological manipulations, it is demonstrated that chronic exposure to opioids is able to produce a lasting enhancement in the mechanisms that support fear learning. This enhancement was found to be independent of changes in pain sensitivity and anxiety, and was also shown not be a consequence of the stress of repeated withdrawal. Moreover, opioid-induced changes in fear learning were accompanied by markers of increased plasticity within the basolateral amygdala, a critical site for associative fear learning. These findings have serious implications for the medicinal and non-medicinal use of opioids, particularly given high rates of opioid prescriptions amongst those who undergo physical traumas.

With respect to receptor systems, although dynorphin release and subsequent kappa receptor activation has been proposed to support increased anxiety as a consequence of drug use, kappa antagonism failed to counter opioid-induced increases in fear learning. Moreover, counter to recent research suggesting anxiolytic properties of kappa antagonism, we were unable to detect any anxiolytic efficacy of a kappa receptor antagonist. It is likely that the conditions under which dynorphin release supports anxiety are limited, and their therapeutic efficacy must be considered in this light.

Lastly, prior traumatic experience was found to robustly increase opioid sensitivity. However, counter to the initial hypothesis, trauma was unable to augment associative learning about opioids in the conditioned place preference task. This finding suggests that the potentiation of amygdala-dependent learning by trauma is restricted to aversive associations. Future research hopes to further explorer the relevance of heightened opioid sensitivity to drug-seeking behaviors.

Major Predictors of PTSD

There are currently several known large-impact risk factors for PTSD, and these risk factors shed light on the disorder's origins. Before addressing drug-abuse comorbidity with PTSD and the potential for drug use to increase the risk for PTSD development, a few of these risk factors will be discussed.

The presence of a pre-existing affective disorder or anxiety disorder carries with it a substantial increase in PTSD vulnerability (Bromet, Sonnega, & Kessler, 1998; Kessler et al., 1995). Moreover, the prior experience of trauma is a reliable predictor of who will develop PTSD in response to a subsequent trauma (Bromet et al., 1998; Kessler et al., 1995). These findings suggest that PTSD is likely to be at least in part a consequence of a general heightening of the physiological systems that support fear and anxiety, and as such, have at least some shared biological overlap with other affective/anxiety disorders. Nevertheless, PTSD is also likely to have unique biological causes. For example, amongst anxiety disorders, alterations in the function and size of the ventromedial prefrontal cortex (vmPFC) appear to be somewhat restricted to PTSD, in contrast to changes in the amygdala, which appears across multiple anxiety disorders (Etkin & Wager, 2007).

Similar to other anxiety disorders, women are approximately twice as likely as men to be diagnosed with PTSD (Breslau, Davis, Andreski, Peterson, & Schultz, 1997; Kessler et al., 1995). Although some of this variance is due to the nature of trauma experienced by women (e.g. victims of sexual assault are most likely to develop PTSD, and sexual assault survivors are more likely to be women), sex differences persist even after one accounts for the nature of trauma experienced (Breslau et al., 1997; Kessler et al., 1995). Whether these differences emerge as a consequence of organizational or activational effects of sex hormones, sex-linked genes, or some other environmental factor is currently unclear. However, it is interesting that heritability estimates for PTSD are higher in women than men (Duncan et al., 2017)

Family and twin studies further suggest that PTSD risk is at least moderately heritable, with heritability estimates between 20-40% (Almli, Fani, Smith, & Ressler, 2014; Banerjee, Morrison, & Ressler, 2017). That being said, the search for causal genes has proven difficult. Although individual genome wide association studies (GWAS) have advanced candidates, these candidates have generally failed to be replicated in independent populations (Banerjee et al., 2017). Indeed, the largest GWAS to date, examining 20,070 individuals, not only failed to confirm previous candidate findings, but failed to find a single gene capable of reaching genome-wide significance across ethnic backgrounds (Duncan et al., 2017). In light of strong evidence that genetic influences do contribute to PTSD, discrepancies between large-scale GWAS may result from differences in the populations studied and the innate difficulty of detecting gene-gene and gene-environment interactions.

As alluded to above, the type of trauma also plays an important role in the development of PTSD. Direct experience of sexual violence carries with it the highest risk for PTSD development (Darves-Bornoz et al., 2008; Kessler et al., 1995; Olaya et al., 2015). Other forms of trauma (e.g. neglect) have a markedly lower risk for PTSD development. Because trauma severity/type predicts the number of individuals that go on to develop PTSD, disease liability

must similarly lie on a continuum, supported by additive/multiplicative biological risk factors. That is, it is likely that anyone could develop PTSD given a traumatic event of sufficient magnitude. However, some individuals are more likely to develop PTSD depending upon the number and type of risk factors they are positive for. Below the hypothesis that opioid use/dependence may serve as an additional risk factor for PTSD is outlined.

Comorbidity Between PTSD and Substance Use Disorders: Prevalence and Directionality

The prevalence of substance use disorder (SUD) amongst individuals with PTSD is of great concern. Individuals with PTSD are two to five times as likely to have an SUD, with some estimates indicating that nearly 40% of individuals with PTSD have an SUD (Chilcoat & Breslau, 1998a, 1998b; Kessler et al., 1995; Merikangas et al., 1998). Given the inherent difficulties of SUD treatment, the presence of a SUD will invariably hinder PTSD treatment, and vice versa. Therefore, understanding the interplay of these conditions is integral.

Traditionally, PTSD-SUD comorbidity has been explained using a negative-reinforcement approach: in response to trauma and subsequent PTSD development individuals attempt to self-medicate with alcohol and other drugs, and in so doing develop a SUD. There is considerable evidence that PTSD does in fact increase the risk for SUD. A longitudinal study following 845 individuals over the course of 5 years found that individuals with an initial diagnosis of PTSD are 4.5 times more likely than individuals without PTSD to develop a SUD (Chilcoat & Breslau, 1998a, 1998b). Moreover, in the United States National Comorbidity Survey of 8098 individuals, it was found that amongst individuals with comorbid PTSD-SUD, PTSD was more likely to have been the first condition to develop (Kessler et al., 1995), further suggesting that PTSD promotes SUD development.

The animal literature has also provided evidence that trauma increases SUD risk. A wealth of studies using stress-induced reinstatement of drug-seeking procedures, in which an acute stressor invigorates a previously extinguished drug-seeking response, demonstrate that stress is able to potently drive the motivation to procure drugs (Mantsch, Baker, Funk, Lê, & Shaham, 2016). Furthermore, animals exposed to either acute or chronic stressors have been shown to display lasting enhancements in drug-seeking (Logrip, Zorrilla, & Koob, 2012; Pizzimenti, Navis, & Lattal, 2017).

In light of both the human and animal literature, there is little doubt that PTSD directly influences the development and persistence of SUDs. However, the extent to which drug use alters PTSD development has been less well studied. Nevertheless, the fact that PTSD predisposes an individual to SUD does not preclude this possibility. Given the dramatic co-occurrence of these two conditions, understanding their bi-directional interaction is of interest.

Of note, there are two ways SUD might increase rates of PTSD. First, an individual with a SUD might be more likely to experience a traumatic event. Indeed, there are some reports that individuals with SUDs are more likely to experience trauma (Bromet et al., 1998; Mills, Teesson, Ross, & Peters, 2006), though data counter to this also exists (Chilcoat & Breslau, 1998b). Second, because not all individuals that experience trauma go on to develop PTSD, it may be the case that the presence of an SUD heightens the likelihood of PTSD development, above and beyond heightened incidence of trauma. There is very limited data on the latter possibility but what findings there are provide modest support for it. In a prospective study of motor vehicle accident victims, it was found that an initial diagnosis of alcohol abuse within the month following the accident was a significant predictor of PTSD at a one year follow-up (Blanchard et al., 1996). Additionally, in the same longitudinal study that found that PTSD predicted SUD development, individuals that had a SUD at baseline and experienced a trauma sometime in the 5-year follow up period had slightly higher rates of PTSD than those that did not

have a baseline SUD but experienced a trauma (14% vs 10%) (Chilcoat & Breslau, 1998a, 1998b). Although this latter difference did not reach statistical significance, sample size was markedly lower than when examining the ability of PTSD to predict SUD development due to exclusion restraints. Therefore, a lack of power may have rendered this increase insignificant.

Admittedly, these studies leave much to be desired. In addition to further studies of humans, animal research that can tightly control the myriad of interacting factors that make human epidemiological studies of this sort so difficult to interpret is well suited for addressing this question. Moreover, as will be described next, it may be the case that certain substances are more likely to alter PTSD vulnerability than others.

Opioid Use/Exposure as a Predictor of PTSD

In conjunction with the widespread increase in opioid sales and use in the past 25 years (Volkow, 2016), the fact that many trauma survivors are prescribed opioids for physical injuries makes understanding the impact they might have on PTSD development and maintenance an important issue. Moreover, there is epidemiological evidence that those that abuse opioids might be especially vulnerable to PTSD development. An Australian survey of 10,641 residents found that amongst individuals with an SUD, individuals with opioid use disorder have the highest rates of PTSD (~33%). This stands in stark contrast to individuals with an alcohol use disorder, who had a rate in line with the general population (~5%) (Mills et al., 2006). These sorts of conditional probabilities cannot address whether or not opioid use causes an increase in PTSD, but this is certainly one interpretation. However, it could alternatively be the case that PTSD does not equivalently influence SUDs: perhaps PTSD influences opioid use disorders more so than alcohol use disorders because of differences in the ease of procurement and stigma surrounding use. Understanding which of these possibilities is true is an important public

health question. The chief experiment in this dissertation demonstrates that chronic opioid exposure is able to interact with trauma to increase fear learning in a rodent model of PTSD.

Using Stress-Enhanced Fear Learning (SEFL) to Model PTSD

The ability to mimic PTSD symptomology in model organisms is paramount to understanding the biological origin of the disorder. The Fanselow Laboratory has developed one such model, called stress-enhanced fear learning (SEFL), which captures multiple facets of PTSD (Perusini et al., 2016; Rau, DeCola, & Fanselow, 2005; Rau & Fanselow, 2009). Because this model will be utilized throughout the experiments in this dissertation, it is discussed below.

In the SEFL model, the ability of a traumatic event to sensitize responses to future stressors is examined. In its basic form, the SEFL model has two components: a traumatic event and a mild stressor that happens sometime later. The traumatic event consists of a series of electric foot shocks delivered randomly in a single session. This trauma is able to produce a robust fear memory for the associated environment, evidenced by the large amount of time that traumatized animals spend freezing when re-exposed to the environment in which the trauma was experienced. At some point after the trauma, animals are then presented with a minor stressor in a novel environment (e.g., a shock or a loud auditory startle stimulus). When placed back in the environment of the minor stressor, animals that had received the trauma display greatly enhanced levels of fear relative to animals that received the minor stressor but did not receive the trauma. There are several notable features about this procedure:

First, there is strong evidence that the trauma in the SEFL model fundamentally changes the mechanisms underlying the acquisition of fear memories, and that increased fear of the context associated with the minor stressor does not merely reflect increased anxiety. Reversing

the order of the trauma and the minor stressor, so that the minor stressor precedes the trauma, mitigates enhanced fear of the environment paired with the minor stressor (Rau et al., 2005). The temporal requirement that trauma must precede the minor stressor in order to augment fear of the associated context indicates that trauma must in some way change the response to the minor stressor. Because traumatized animals do not show elevated unconditional responses to the minor stressor – indeed, we have found that traumatized animals actually show smaller motor responses to the minor shock after trauma (Poulos et al., 2014) – increased fear of the context paired with the minor stressor has been hypothesized to reflect augmented associative learning. That is, it is not the averseness of the mild stressor that is different in traumatized animals, but the extent to which the aversive experience shapes subsequent behavior. Hence, the name "stress-enhanced fear learning."

Second, the sensitization of fear learning is long lasting and independent of the ability to recall the traumatic experience. Increased fear learning occurs for at least 90 days after the initial trauma (Rau & Fanselow, 2009). Moreover, extinguishing fear of the trauma context does not alter the ability of the trauma to enhance fear learning (Rau et al., 2005); administration of the trauma to young animals that are unable to form contextual fear memories still produces augmented fear learning in adulthood (Poulos et al., 2014); and intracerebroventricular administration of the NMDA receptor antagonist APV prior to trauma, which results in a failure of traumatized animals to display fear of the trauma context, nevertheless display enhanced fear learning (Rau et al., 2005). These findings are striking in that they suggest that the sensitization of fear learning by trauma occurs separate from the memory for the traumatic event. Therefore, reducing fear of trauma-associated stimuli does not necessarily mean that an animal's fear systems are no longer sensitized. This is relevant to the treatment of PTSD because the primary focus of treatment has been the extinction of responses to trauma-associated stimuli. The ability of the SEFL model to capture both associative consequences of traumatic

experience (i.e. the memory for the traumatic event) and non-associative consequence of traumatic experience (i.e. those that do not require memory of the traumatic event) makes it a powerful tool. Moreover, because one of the major predictors of developing PTSD is a prior history of traumatic experience, the SEFL model may capture biological variance related to this risk factor as well.

Third, the SEFL model is able to capture several other facets of PTSD. For instance, animals exposed to the traumatic experience used in the SEFL procedure show increased anxiety in the elevated plus maze and open field (Perusini et al., 2016), increased depressive-like behavior in the forced swim test (Perusini et al., 2016), increased reactivity to a startle stimulus (Perusini et al., 2016), and have been found to have altered glucocorticoid cycling (Poulos et al., 2014), all of which are consistent with PTSD. Thus, the SEFL model lends itself to studying a wide variety of responses to trauma using a common set of procedures. Moreover, the ease of implementing these procedures and the robust nature of the effects produced makes it ideal for probing the biology of PTSD.

Biological Contributors to SEFL and Their Link to PTSD:

Neuroimaging studies have implicated several brain regions in the pathophysiology of PTSD. Most consistently these regions have included the amygdala, medial prefrontal cortex (mPFC) and hippocampus (Rauch, Shin, & Phelps, 2006; L. M. Shin & Liberzon, 2010).

The basolateral amygdala (BLA) is an integral part of the circuit responsible for generating fear and is of particular relevance to the sensitization of fear learning following trauma. The BLA is anatomically well situated to integrate peripheral sensory information in the service of fear memory acquisition, receiving auditory, taste, visual, and somatosensory inputs. As a consequence of synaptic plasticity emerging from this convergence, the BLA is thought to be capable of imbuing sensory stimuli with affective salience in order that they can generate the

subjective, autonomic and behavioral responses to threat that we call fear (Davis, 1992; M. S. Fanselow & Gale, 2003; M. S. Fanselow & Pennington, 2017; Gray, 1993; Kim & Fanselow, 1992; Koenigs & Grafman, 2009; LeDoux, 2003; Maren & Fanselow, 1996; Rauch et al., 2000). Indeed, damage to the BLA dramatically reduces fear and anxiety in both humans and model organisms (M. S. Fanselow & Gale, 2003; Feinstein, Adolphs, Damasio, & Tranel, 2011; Maren, Aharonov, & Fanselow, 1996), and manipulations that block synaptic plasticity within the BLA similarly block fear learning, though not necessarily the expression of fear (M. S. Fanselow & Kim, 1994; Rumpel, LeDoux, Zador, & Malinow, 2005; Schafe & LeDoux, 2000). Moreover, functional imaging studies indicate that patients with PTSD demonstrate exaggerated activity in the amygdala in response to negative stimuli (Bechara et al., 1995; Cahill, Babinsky, Markowitsch, & McGaugh, 1995; Rauch et al., 2000; L.M. Shin, Rauch, & Pitman, 2006), suggesting that increased associative learning processes within the BLA may underlie PTSD. Following from this, the contributions of the BLA to SEFL have been examined.

Initial studies examined the expression of proteins necessary for excitatory neurotransmission in the BLA following trauma because this sort of change would parallel findings of increased amygdala excitability in PTSD patients. Here, it was found that animals that underwent the SEFL trauma had increases in the GluA1 subunit of the AMPA receptor within the BLA, but not the GluA2 subunit of the AMPA receptor, or the NR1 subunit of the NMDA receptor (Perusini et al., 2016). Yet to be published work indicates that this increase in GluA1 protein level is long-lasting, persisting for at least two weeks after trauma. Notably, AMPA receptors are typically a heteromer of the GluA1 and GluA2 subunits, but when GluA1 subunit availability exceeds that of GluA2, monomeric GluA1-only AMPA receptors are assembled (Wiltgen et al., 2010). These monomeric GluA1 receptors are Ca^{2+} permeable and an increase of these Ca^{2+} permeable glutamate receptors leads to an increased capacity for long-term potentiation (Mahanty & Sah, 1998; Wiltgen et al., 2010). As such, the increase in GluA1 protein levels may explain enhanced BLA dependent learning in traumatized animals.

The actions of corticosterone, a canonical stress hormone, on the BLA have also been implicated in the genesis of SEFL. The SEFL trauma has been shown to produce lasting increases in glucocorticoid receptor expression in the BLA, but not the mPFC or hippocampus (Poulos et al., 2014). Furthermore, the SEFL trauma produced lasting changes in the diurnal cycle of circulating glucocorticoids (Poulos et al., 2014), a finding consistent with PTSD patients (Yehuda, 2009). Demonstrating a causal role for glucocorticoids in SEFL, intraperitoneal injections of the glucocorticoid synthesis blocker metyrapone prior to trauma was able to block the subsequent enhancement in fear learning – as well as the increase in GluA1 protein levels in the BLA (Perusini et al., 2016). Going further, direct infusions of the glucocorticoid receptor antagonist mifepristone prior to trauma was also able to block SEFL (Perusini et al., 2016). As such, it has been postulated that an increase in glucocorticoid release into the BLA during trauma mediates a lasting increase in GluA1 protein expression in the amygdala which in turn produces a heightened ability to undergo fear learning (Perusini et al., 2016). Interestingly, acute glucocorticoid release in response to trauma has been shown to negatively predict the development of PTSD (McFarlane, Atchison, & Yehuda, 1997; Resnick, Yehuda, & Acierno, 1997; Resnick, Yehuda, Pitman, & Foy, 1995). In conjunction with findings of altered gluccoorticoid cycling in PTSD patients – as opposed to a general heightening of release – the link between glucocorticoids and PTSD is unlikely to be a simple “more is bad” relationship.

Although the induction of SEFL is unlikely to solely require the BLA, studies to date have failed to find robust alternative contributors to SEFL. For instance, because reductions in the size and function of the vmPFC have consistently been implicated in PTSD, I previously examined the impact of vmPFC lesions on SEFL (Pennington, Anderson, & Fanselow, 2017). vmPFC lesions failed to consistently impact SEFL, and when they did, changes appeared to reflect changes in contextual processing as opposed to fear sensitization (Pennington et al., 2017). Similarly, although the hippocampus seems to play an important role in the acquisition of fear memories when these memories involve multidimensional stimuli like locations, they have

little impact on fear of discrete unidimensional stimuli such as tones (Kim & Fanselow, 1992). Blockade of NMDA receptors within the hippocampus during trauma also fails to block SEFL (Rau et al., 2005), indicating that the lasting changes that support SEFL are unlikely to require plasticity within the hippocampus. Thus, although the vmPFC and hippocampus may play an important role in PTSD, they are less likely to factor into the sensitization of fear learning that is seen following trauma. We have alternatively proposed that changes in these regions contribute to altered generalizability of fear memories in PTSD patients, wherein diminished contextual processing leads fear memories to be expressed in inappropriate situations. In light of this, studies in this dissertation pertaining to how opioid use impacts SEFL have focused primarily on the BLA. This is not to say that drug use and stress interact solely in the BLA; it was merely a logical starting point from which to build upon prior research.

The Intersection of Opioid Pharmacology and Fear

Endogenous and exogenous opioids produce widespread physiological effects via their actions on mu, delta, and kappa opioid receptors, which are distributed throughout much of the nervous system (Le Merrer, Becker, Befort, & Kieffer, 2009). This influence is not exclusive of the neural circuits that support fear, and thus, the direct impact that opioids may have on these circuits is of interest. After examining the impact of chronic morphine administration and withdrawal on fear learning, experiments in this dissertation attempt to ascertain the molecular pathways leading from opioid use to fear sensitization. Because the mu and kappa opioid receptors are of particular relevance to how opioids might come to alter fear and anxiety, and because these are the receptors targeted in the presented experiments, they are discussed below.

Mu opioid receptors are the primary target of the most commonly used opioid analgesics, with morphine derivatives and synthetic opioids alike having their highest affinity for this receptor (Emmerson, Liu, Woods, & Medzihradsky, 1994; Mignat, Wille, & Ziegler, 1995).

Moreover, although delta and kappa receptors have both been demonstrated to have analgesic properties (Holdridge & Cahill, 2007; Von Voigtlander & Lewis, 1982), much of the addictive properties of opioids can be attributed to the mu opioid receptor. This follows from the findings that mu opioid receptor knockout mice are insensitive to the rewarding effects of opioid analgesics and will not self-administer opioids (Cui et al., 2014; Matthes et al., 1996). Mu opioid receptors are not only necessary for opioid reward, but are also necessary for the ability of chronic opioid treatment to produce physiological dependence: mu opioid receptor knockout mice do not demonstrate signs of opioid withdrawal when treated chronically with opioids (Cui et al., 2014; Matthes et al., 1996). Notably, this is not because opioid receptor activation directly precipitates withdrawal – in fact, the opposite is true: opioid receptor antagonism induces withdrawal – but rather, opioid dependence and withdrawal result from downstream physiological changes the body invokes to counter chronic mu opioid receptor agonism. Nevertheless, mu opioid receptor activation is clearly able to influence both positive and negative affective states, be it as a consequence of direct or indirect influences.

The ability of chronic mu opioid receptor activation to induce negative affective states and potentiate aversive learning may depend upon their expression within limbic circuitry that support aversive learning. Mu opioid receptors are richly expressed within the BLA, although given a general paucity of *mu* mRNA within the BLA, these receptors are likely to be located on presynaptic afferents (Mansour, Fox, Burke, et al., 1994; Mansour, Fox, Thompson, Akil, & Watson, 1994). Because mu opioid receptors are inhibitory g-protein coupled receptors (as is the case for all opioid receptors), terminal expression inhibits neurotransmitter release (Al-Hasani & Bruchas, 2011; Le Merrer et al., 2009). Chronic use and subsequent receptor desensitization/internalization might therefore oppose this process and upon opioid cessation lead to augmented excitatory drive onto the BLA, which could in turn facilitate fear. Alternatively, mu receptors are also expressed on the intercalated cells of the amygdala as well

as the periaqueductal gray (Mansour, Fox, Burke, et al., 1994; Mansour, Fox, Thompson, et al., 1994), both of which are central to the expression of fear behaviors.

Dynorphin signaling through kappa receptors is another potential mechanism through which chronic opioid exposure could sensitize fear learning. In contrast to the mu receptor, which is endogenously activated by endorphins, the kappa receptor is instead activated by the peptide dynorphin (Chavkin, James, & Goldstein, 1982; Chavkin & Koob, 2016; James, Chavkin, & Goldstein, 1982). Like mu opioid receptors, kappa activation has been demonstrated to be analgesic, increasing pain thresholds (Von Voigtlander & Lewis, 1982). However, kappa receptor agonists have also been found to be highly aversive (Bals-Kubik, Ableitner, Herz, & Shippenberg, 1993; Contarino & Papaleo, 2005; Land et al., 2008) and kappa antagonists have shown benefit as antidepressants and anxiolytics in pre-clinical models (Chartoff et al., 2012; Knoll, Meloni, Thomas, Carroll, & Carlezon, 2007; McLaughlin, Li, Valdez, Chavkin, & Chavkin, 2006; McLaughlin, Marton-Popovici, & Chavkin, 2003). Thus, in contrast to the rewarding impact of endorphin signaling through the mu opioid receptor, dynorphin signaling through the kappa receptor represents a predominantly aversive side of opioid receptor signaling.

The relevance of changes in dynorphin/kappa signaling have been of particular interest in the treatment of addiction. Koob and colleagues have advanced the hypothesis that negative aversive states associated with withdrawal, and the subsequent drive to escape withdrawal, is mediated in part through kappa receptor activation (Bruchas, Land, & Chavkin, 2010; Schlosburg et al., 2013). This hypothesis was spurred by findings of elevated levels of kappa binding and dynorphin signaling in cocaine addicts (Hurd & Herkenham, 1993), increased levels of dynorphin and prodynorphin in the striatum of animals that self-administered heroin (Hurd, Brown, Finlay, Fibiger, & Gerfen, 1992; Schlosburg et al., 2013), and kappa antagonism being found to reduce escalation of heroin self-administration and stress-induced drug-seeking in animal models (Beardsley, Howard, Shelton, & Carroll, 2005; Graziane, Polter, Briand, Pierce, &

Kauer, 2013; Schlosburg et al., 2013). Moreover, kappa receptor knockout mice show a reduction in negative depressive-like states following chronic heroin administration (Lutz et al., 2014). Thus, whereas acute mu opioid receptor activation supports the initial hedonic impact of opioid use, perhaps increased kappa receptor activation as a consequence of continued drug use precipitates negative affective states. Blocking these negative affective states might aid in addiction treatment, and moreover, reduce their impact on comorbid conditions including depression and PTSD.

Much of the work examining the impact of kappa receptor signaling on affective states has centered on the nucleus accumbens and striatum, wherein kappa antagonism produces antidepressant-like effects (Carlezon et al., 2006; Donahue et al., 2015; Ebner, Roitman, Potter, Rachlin, & Chartoff, 2010; Newton et al., 2002; Zan et al., 2015). However, the striatum plays little role in the acquisition and expression of fear and is therefore an unlikely locale for dynorphin release to alter fear. That being said, kappa receptors are also located in both the amygdala and bed nucleus of the stria terminalis (BNST), which play critical roles in the acquisition and expression of fear memories (Davis & Walker, 2013; M. S. Fanselow & Kim, 1994; Gale et al., 2004; Sullivan et al., 2004; Waddell, Morris, & Bouton, 2006). Indeed, antagonism of kappa receptors in the BLA has been shown to be anxiolytic (Bruchas, Land, Lemos, & Chavkin, 2009), as is a loss of pre-synaptic kappa receptors on BLA-to-BNST efferents (Crowley et al., 2016). Still, the impact of chronic opioid administration and withdrawal on these receptors, and how such changes might influence fear learning, has not been examined.

In the experiments contained within this dissertation, I will assess the impact of kappa receptor antagonism on the ability of chronic morphine administration to augment fear learning. By determining the systems through which chronic opioid exposure and withdrawal potentiates fear learning, pharmacological targets for intervention might be discovered.

Beyond SEFL: Does Trauma Enhance BLA-Dependent Drug Memories?

Using epidemiological methods to examine SUD-PTSD comorbidity, it has been found that PTSD confers risk for developing an SUD (Chilcoat & Breslau, 1998a, 1998b; Kessler et al., 1995; Merikangas et al., 1998). This parallels a vast body of research showing that stress during rodent development augments both responses to drugs of abuse and drug self-administration in adulthood, as well as research finding that acute stressors are able to robustly drive drug-seeking and -taking behaviors in rodents (Logrip et al., 2012; Mantsch et al., 2016; Pizzimenti et al., 2017). One might presuppose that these findings support a self-medication hypothesis, wherein individuals with PTSD use drugs and alcohol in order to lessen their symptoms. Furthermore, it could be assumed that SUD liability at this point is limited to those individuals who already had a predisposition for developing addiction. That is, PTSD merely leads individuals to use drugs but does not alter the propensity to develop a SUD once drug use commences. An alternative view, and the one to be explored in this dissertation, is that the experience of trauma fundamentally changes the way in which the brain processes drugs of abuse, generating not merely the desire to use drugs to escape symptoms, but to augment associative learning processes that propel SUD development. This hypothesis is supported by the finding that early life stress in rodents is capable of increasing morphine and cocaine sensitization in adulthood, a non-operant form of learning that is thus not amenable to a negative reinforcement hypothesis (Kalinichev, Easterling, & Holtzman, 2002; Kikusui, Faccidomo, & Miczek, 2005). Moreover, in a recent study of the ability of the SEFL trauma to alter drug self-administration, it was found that prior trauma did not alter the rate of lever-pressing for methamphetamine under multiple schedules of reinforcement, nor did it impact the rate at which drug seeking responses extinguished (Pizzimenti et al., 2017). However, drug associated cues had a much greater ability to reinstate drug-seeking after extinction (Pizzimenti

et al., 2017). These findings suggest that trauma increases the impact stimuli associatively paired with drugs of abuse have on an individual without necessarily influencing motivation to procure drugs independent of these cues. We propose that some of the molecular changes in the emotional hubs of the brain involved in the development of PTSD symptomology following trauma (i.e., the amygdala) similarly support an increased proclivity to develop SUDs. More specifically, it will be tested whether drug-associated learning that is dependent upon the BLA is similarly potentiated by trauma.

The BLA not only supports the acquisition of aversive memories but is also important for the acquisition and expression of appetitive associations and drug-taking behaviors relevant to SUDs. Lesions of the BLA impair the expression of conditioned place preference (CPP) for both food and cocaine, in which a location must be associated with a reward (Everitt, Morris, O'Brien, & Robbins, 1991; Fuchs, Weber, Rice, & Neisewander, 2002). Similarly, lesions of the BLA reduce the ability of animals to associate their actions with specific rewards (Balleine, Killcross, & Dickinson, 2003; Johnson, Gallagher, & Holland, 2009). Demonstrating the need for amygdala-dependent plasticity in the formation of appetitive associations, NMDA receptor antagonists infused into the amygdala during learning block the acquisition of lever-pressing for a food reinforcer (Baldwin, Holahan, Sadeghian, & Kelley, 2000). With respect to the BLA's role in drug-taking behaviors, lesions of this region impair the ability of animals to work for cocaine under second-order schedules of reinforcement (Whitelaw, Markou, Robbins, & Everitt, 1996), infusions of dopamine antagonists into the amygdala reduce drug self-administration (Di Ciano & Everitt, 2004), and inactivation of the amygdala blocks the ability of both drugs and drug-associated cues to reinstate extinguished drug-seeking responses (Fuchs & See, 2002). Furthermore, several reports indicate that cues associated with drugs of abuse are able to strongly increase amygdala activity in human drug users (T. R. Franklin et al., 2007; Kilts et al.,

2001). Collectively, these studies show that the BLA plays an important role in the acquisition and expression of appetitive associations related to drug consumption.

In summary, the BLA supports both associative fear learning and associative learning about drugs of abuse. However, although the SEFL trauma is known to sensitize associative learning mechanisms in the BLA necessary for fear learning, and that stress is able to alter drug-taking behaviors, it is unclear whether trauma is able to influence BLA-dependent associative learning. Therefore, in this dissertation I explore the possibility that trauma produces lasting increases in conditioned place preference for an opioid, a BLA dependent process. This work could not only shed light on how trauma impacts BLA-dependent learning processes, but the mechanism through which trauma alters the progression from drug use to drug dependence.

Outline of the Contained Work

Above it has been outlined how SUDs and PTSD are highly comorbid, that those with an opioid use disorder may be particularly liable to developing PTSD, and that this increase this may stem from the direct action of opioids on fear circuitry. In **"Section 1: Opioid-induced Enhancements in SEFL: Cognitive-Behavioral Processes and Neuronal Correlates**," experiments are presented that demonstrate the ability of chronic opioid exposure to enhance fear learning, and moreover, that these changes are accompanied by markers of enhanced synaptic plasticity within the BLA. In **"Section 2: Contributions of Kappa Signaling to Morphine-Induced Enhancements in SEFL,"** experiments are presented which show an inability of kappa receptor antagonism to ameliorate opioid-induced increases in SEFL, along with other indices of anxiety and depression. Lastly, in **"Section 3: Potentiation of Opioid Sensitivity by Prior Traumatic Experience,"**

a study examining the ability of trauma to augment a form of BLA-dependent associative learning about opioids is described. Trauma is shown to augment subsequent responses to opioids.

Methods

Animals

For all experiments, male C57 BL/6J mice, between 2 and 3 months of age, were obtained from Jackson Laboratories. Mice were housed individually in disposable mouse cages (Innovive, San Diego CA) on a ventilated rack in a temperature-controlled vivarium for at least 2 weeks prior to commencing experiments. The lighting schedule was 12 hours on, 12 hours off, with lights on at 8 a.m. The Chancellor's Animal Research Committee at UCLA approved all animal testing procedures.

Experiment Group Sizes (Total n=347):

Experiment 1: Total: n=29. No Trauma–Saline: n=8; No Trauma–Morphine: n=8; Trauma–Saline: n=6; Trauma–Morphine: n=7.

Experiment 2: Total: n=24. For EPM, Saline: n=12; Morphine: n=12. For shock reactivity assessment, half of each group from EPM were tested. The other half were euthanized after EPM for immunohistochemical analysis.

Experiment 3: Total: n=28. Once-Daily–Saline: n=6; Twice-Daily–Saline: n=6; Once-Daily–Morphine: n=8; Twice-Daily–Morphine: n=8.

Experiment 4: Total: n=27. Placebo–Saline: n=7; Placebo–Naltrexone: n=7; Morphine–Saline: n=6; Morphine–Naltrexone: n=7.

Experiment 5: Total: n=50. No Trauma–Saline: n=12; No Trauma–Morphine: n=13; Trauma–Saline: n=13; Trauma–Morphine: n=12.

Experiment 6: Total: n=16. Trauma–Saline: n=8; Trauma–Morphine: n=8.

Experiment 7: Total: n=16. For c-Fos, Homecage: n=4 (2 saline, 2 morphine); Saline: n=6; Morphine: n=6. For GluA1, only non-Homecage animals were assessed because exposure was

found to alter expression of GluA1 and there were too few subjects to statistically control. Animals from Experiment 7 were the same as those from Experiment 2.

Experiment 8: Total: n=27. For c-Fos and initial GluA1 analysis, n=15; Homecage: n=4 (2 saline, 2 morphine); Saline: n=5; Morphine: n=6. For GluA1, only non-Homecage animals were assessed because exposure was found to alter expression of GluA1 and there were too few subjects to statistically control. For expanded GluA1 analysis relating levels to behavior, n=23; Saline=9, Morphine=14. Animals from experiment 8 were saline-treated animals from Experiment 9.

Experiment 9: Total n= 99; For Effect of JDTic on morphine-induced changes in SEFL, n=87; Saline–Saline: n=20; Saline–JDTic: n=20; Morphine–Saline: n=23; Morphine–JDTic: n=24. For assessment of JDTic's ability to block kappa agonism, n=12; Saline=6, JDTic=6.

Experiment 10: Total n=16. Saline: n=8; JDTic: n=8.

Experiment 11: Total: n=15. No Trauma: n=8; Trauma: n=7.

Drug Treatments

Chronic Morphine Injections:

Across experiments, animals were administered a common escalating regimen of twice daily morphine sulfate (National Institutes of Drug Abuse, NIDA; Bethesda MD), or an equivalent volume of saline, over the course of 8 consecutive days. The dose of each injection on successive days was as follows: Day 1 = 10 mg/kg; Day 2 = 20 mg/kg; Day 3 = 30 mg/kg; Day 4 = 40 mg/kg; Days 5-8 = 50 mg/kg. Morphine sulfate was dissolved in sterile saline and injected subcutaneously at a volume of 10 ml/kg. Injections were administered daily between the hours of 8-10 a.m. and 5-7 p.m.

Morphine Pellet Implantation and Repeated Naltrexone-Precipitated Withdrawal:

Morphine pellets containing 8.3 mg of morphine, or placebo pellets, were subcutaneously implanted. A 1-inch area just below the nape of the neck was shaved and sterilized prior to making a 0.8 cm vertical incision and imbedding the pellet doubly wrapped in sterilized nylon mesh. The wound was then closed with nylon sutures and a mixture of bupivacaine (0.5%) and topical antibiotic was applied to the site of the wound. Pellets containing 25 mg morphine were obtained from NIDA and cut down to the appropriate weight, and placebo pellets were treated similarly

In order to produce cycling of morphine withdrawal in pellet-implanted mice, mice were treated with either naltrexone (0.25 mg/kg, i.p.; obtained from Sigma), or saline, twice daily, for 7 days, beginning the day after pellet implantation. The efficacy of naltrexone-precipitated withdrawal was assessed in the morning of days 1, 3, 5, and 7, post-implant. Animals were placed in a translucent plexiglass cylinder (15.24 cm wide by 38.1 cm tall) immediately after saline/naltrexone injection for 15 minutes. The plexiglass cylinder was set atop a piece of clean cloth that was weighed before and after the 15-minute session to assess defecation. Additionally, the number of jumps observed in the final 10 minutes of each session were counted.

JDTic in Conjunction with Chronic Morphine Injections:

The long-acting kappa opiate receptor antagonist, atrans-(3R,4R)-dimethyl-4-(3-hydroxyphenyl) piperidine (JDTic), was dissolved in sterile saline and administered at a dose of 10 mg/kg (injected at 10 ml/kg, i.p.). JDTic was generously provided by Chris Evans at UCLA. Although antagonism by JDTic is sufficient to non-competitively block activation of kappa

receptors for 2-3 weeks (Bruchas et al., 2007), JDTic was administered every 3-4 days when given in combination with morphine in order to counteract the potential for up-regulation during morphine exposure/withdrawal. More specifically, JDTic was administered 12 hours before commencing the chronic morphine regimen, on the fourth and eighth days of that regimen, and then 3 and 6 days into withdrawal. Additionally, in order to confirm the ability of JDTic to produce lasting antagonism of the kappa opioid receptor, a set of animals was treated with either saline or JDTic (10 mg/kg, i.p.). 3 days later, animals were given a baseline locomotor session in which they were placed in a locomotor chamber for 30 minutes. Across the next two days, animals' activity in response to an injection of the kappa agonist U-50488H (10 mg/kg, i.p.; obtained from Sigma), and then saline, were assessed in 30 minute sessions. Locomotion was assessed in empty plastic cages measuring 17.8 cm wide and 29.2 cm long. Overhead video recordings were analyzed using EthoVision (Noldus, Wageningen, The Netherlands) to obtain distance travelled.

Behavioral Testing

Fear Conditioning Apparatus:

All fear conditioning procedures took place in Med Associates conditioning chambers (VFC-008; 30.5 x 24.1 x 21 cm), controlled by Med Associates Video Freeze software (Med Associates, St. Albans VT). Chambers were configured to represent distinct contexts, differing in physical appearance, luminosity, odor, and background noise. Transport to the different contexts was also varied to aid in discriminability: animals were transported to one context in their home cage and to the other in a separate opaque box with sawdust bedding. Scrambled shocks were delivered to grid floors in the chambers via Med Associates shock scramblers (ENV 414-S). Sessions were recorded by near infrared cameras and freezing and motion were measured using Med Associates Video Freeze software. Using this software, motion was

calculated as the average number of pixels whose grey scale value changed per frame (30 frames/second) during a specified time. Freezing was defined as motion below a threshold that conformed to visual inspection of behavior, lasting at least 1 second (Perusini et al., 2016; Poulos et al., 2014; Poulos, Zhuravka, Long, Gannam, & Fanselow, 2015; Zelikowsky, Bissiere, & Fanselow, 2012; Zelikowsky, Bissiere, et al., 2013; Zelikowsky, Hast, et al., 2013).

SEFL Procedure:

The SEFL procedure assesses enhanced fear learning following exposure to a traumatic event (Rau et al., 2005; Rau & Fanselow, 2009), and took place across 4 days (See Figure 1A). Prior to this, all animals were habituated to being handled for 3 days, 30-60 sec/day, and were also habituated to transport from the vivarium to the laboratory for 2 days, for 15 minutes/day, in their home cage. On the first day of the procedure, animals experienced the traumatic stressor, consisting of 10, 1 mA, 1 second shocks, pseudo-randomly distributed over the course of an hour in a distinctly configured conditioning chamber/context. Non-trauma animals were placed in the context for an equivalent amount of time but were not shocked. Shock sensitivity was assessed by examining average motion during shock periods, and freezing throughout trauma was assessed during 30 second intervals, beginning 30 seconds after each shock. On the second day, animals were re-exposed to the context of the traumatic stressor for eight minutes to assess their memory of the traumatic event. Freezing was assessed throughout. On the third day, animals were exposed to a mild stressor in a novel environment. After 3 minutes exploring the novel chamber/context, animals were given a 0.5 mA, 2 second, shock. They were taken out of the chamber 2 minutes later. Shock reactivity and freezing before/after the shock were assessed. On the fourth day, animals were placed back in the context of the mild stressor for 8 min and freezing was assessed.

Shock Sensitivity:

In order to obtain a parametric assessment of shock sensitivity, animals were exposed to shocks of increasing intensity during a single session. Each shock was 2 sec long, and was separated by 1 min. Shocks ranged from 0.1-0.5 mA, in 0.05 mA increments. Each shock intensity was repeated twice, in succession. Motion during the shocks as well as during an equivalent pre-shock period were measured.

Elevated Plus Maze:

The elevated plus maze (EPM) had 4 intersecting arms, each measuring 29.2 x 7.6 cm, suspended 53.3 cm above the floor. The two opposing enclosed arms had opaque walls that were 14.5 cm tall along their length. The elevated plus maze was located in a well-lit room. Behavior was recorded by a camera suspended above the maze. Time spent in the open/closed arms and distance travelled was assessed using EthoVision (Noldus, Wageningen, The Netherlands). Following 2 days of transport habituation to a room adjacent to where the elevated plus maze was located, animals were tested by being placed in the central portion of the maze and allowed to freely explore for 5 min. Percent of time in the open arms, and total distance travelled, were measured.

Forced Swim Test:

The forced swim test took place in a translucent plexiglass tank, 30.5 cm tall and 19.4 cm in diameter. This tank was filled with approximately 3.5 L of 30 ° C H20, which stood 12 cm from the bottom of the tank.

We adopted a two-day forced swim task that was previously shown to be sensitive to kappa antagonism and prodynorphin gene deletion (McLaughlin et al., 2003). On the first day,

animals were placed in the tank for 15 minutes. On the second day, animals were placed in the tank 4 different times, each occurrence being 6 minutes in duration and separated by 10 minutes. Animals were dried with paper towels and returned to their home cage after each session in the water.

In accordance with previous reports, we scored the amount of time animals were immobile during the last 4 minutes of each placement in the water. To do so, videos were viewed by a blind observer at a 50% playback speed and immobility was scored using a time sampling procedure, such that animals were assessed for immobility every 4 seconds. Immobility was defined as the complete absence of any paddling movements of the legs.

Conditioned Place Preference:

Conditioned place preference (CPP) was carried out in a square plexiglass chamber with white walls (26.67 cm wide and 17.8 cm high), divided centrally by a black plexiglass wall. The central wall had a passageway that was 5.1 cm wide and 6.35 cm tall providing access between the two sides of conditioning chamber; a translucent plastic insert could bar off this passageway. The sides of the chamber were differentiated by flooring: one was composed of parallel bars, each 3.15 mm in diameter and spaced 4.8 mm apart; the other composed of cross-hatched bars, each 0.8 mm in diameter and spaced 5.5 mm apart. The room external to the chamber was dimly lit. Between sessions the chamber was cleaned with a 50% windex/water solution. A camera located below the chambers monitored behavior.

The place preference procedure occurred across 8 days. During the first two days, animals were acclimated to the chamber by allowing them free access to both sides of the chamber during 30 min (first day) and 15 min (second day) sessions. The second of these acclimation sessions served as the pre-test session and side preference in this session was

used to assign which chamber animals would receive morphine in. Assignment was done so that within each group there was not a pre-training preference for the morphine paired side. Subsequently, across 4 days, animals were alternately confined to one of the two chambers for 45 min, immediately after being injected with either saline or morphine (20 mg/kg, i.p.). Morphine and saline were given in alternating sessions and which was received first was counterbalanced. On the 7th day, animals were placed in the chamber and allowed to freely choose between chamber sides during a 15 min session. On the 8th day, animals received a second 15 minute test, 5 min after being injected with morphine (20 mg/kg, i.p.).

In order to reduce the stress associated with morphine injection and transport to the place preference room, animals were handled for 5 days prior to beginning place preference training, including being habituated to restraint on the last 3 days, and transport to the conditioning room on final day. Moreover, 30 minutes after the two acclimation sessions animals were given i.p. injections of saline.

Tissue Collection, Immunohistochemistry and Cell Counts

For immunohistochemical staining, brains were rapidly extracted 90 minutes after behavior and placed in cold 4% paraformaldehyde overnight before being transferred to 30% sucrose in 1X PBS. Once brains had sunk, tissue was frozen at -80 °C prior to being sectioned at 40 microns and collected in PBS.

For c-fos staining, tissue was first incubated overnight at 4 °C in a blocking solution (3% normal goat serum, 0.3% Triton X-100, 1X PBS) containing a polyclonal rabbit anti c-fos antibody (1:10,000; Millipore: ABE457). Tissue was then washed 3x in 1X PBS prior to being incubated in blocking solution containing goat anti-rabbit Alexa 594 antibody for 2-4 hours

(1:500; Thermo Fisher Scientific: A-11012). Tissue was then washed in PBS, mounted on slides, and coverslipped with Vectashield with DAPI (Vector Laboratories, Burlingame CA).

GluA1 staining was performed using a nearly identical protocol to c-fos except for the primary antibody (Abcam Rabbit Anti Glutamate Receptor 1: AB31232. 1:1000 dilution). Additionally, a 45-minute blocking step preceded incubation in primary antibody.

Multi-channel images were taken using a Keyence BZ-X710 fluorescent microscope at 4x-10x magnification. All images within a brain region/experiment were captured using identical microscope/camera settings.

After c-fos staining and image acquisition, images were subsequently processed using an automated cell counting procedure developed in-house using Image J software (imagej.nih.gov). In brief, after converting images to grayscale, a background subtraction procedure using a rolling ball radius equal to the maximum radius of any cell observed was performed to account for differences in background fluorescence between images. Subsequently, images were thresholded and a watershed procedure was used to separate adjoining cells. Cells within a region of interest were then counted utilizing Image J's particle analysis function with minimum/maximum particle size criteria to exclude artifacts. All parameters were calibrated to a sample of manually counted images such that the automated procedure yielded >90% congruent results in cell counts/location. The same parameters were applied to all images. Regions of interest were traced manually from DAPI channel images to reduce bias.

For GluA1 image analysis, after grayscale conversion, fluorescent intensity was calculated within the region of interest for several images for each animal. The average intensity across images was then calculated, weighted by the area of the region of interest in each image. When animals from different batches of immunohistochemistry were combined, average intensity values from each batch were first z-scored and z-scores were then analyzed.

Images of the central nucleus of the amygdala (CEA) and BLA were taken between -1.2 and -2.2 mm relative to bregma, according to the atlas of Franklin and Paxinos (K. B. J. Franklin & Paxinos, 2008). Per animal, 8 to 20 images of each region were taken and counts were normalized to the cumulative surface area of the region of interest across images. Images of the BNST were taken between 0.38 to -0.1 relative to bregma, focusing on the dorsal BNST. Per animal, 6-12 images were taken.

Statistical Analysis

Data were analyzed using SPSS v22. For multifactorial designs, omnibus ANOVA were initially performed, followed by analysis of simple interactions when higher order interactions were present, followed by Bonferroni-corrected post-hoc comparisons. For unifactorial ANOVA, orthogonal contrasts were used when groups could be segregated in an *a priori* manner (e.g., in c-Fos analyses, where home cage controls are first compared to experimental groups, and experimental groups are then compared). For repeated measures ANOVA, when sphericity was violated, the Greenhouse-Geisser correction was used. Nevertheless, unadjusted degrees of freedom are presented for ease of identifying group sizes; p values reflect correction for sphericity. Multiple regression was used to predict freezing from GluA1 z-scores and morphine treatment group membership, dummy-coded such that Saline=0, Morphine=1.

Results

Section 1:

Opioid-induced Enhancements in SEFL: Cognitive-Behavioral Processes and Neuronal Correlates

Experiment 1: Chronic morphine exposure and withdrawal potentiates SEFL

The impact of chronic opioid exposure on fear sensitization was first assessed using the SEFL procedure, which captures the sensitization of fear learning observed following traumatic experience (depicted in Figure1A). Here, it was found that a history of prior opioid exposure exacerbates the ability of trauma to potentiate fear learning (Figure 1).

Over the course of 8 days, animals received twice daily injections of saline or escalating doses of morphine, ranging from 10-50 mg/kg. During this time, morphine-treated animals displayed a substantial drop in weight relative to saline-treated animals (Day x Morphine Interaction: $F_{7,189}$=46.37, p<0.001), reducing to ~89% of their baseline weight by the last day of morphine administration (Figure 1B). Then, during the week-long abstinence period prior to behavioral testing, the weight of morphine-treated animals steadily recovered (Day x Morphine Interaction: $F_{6,162}$=36.25, p<0.001), such that morphine and saline-treated animals did not differ by the time of the traumatic event (Figure 1B. t_{27}=1.75, p=0.09). This was taken as a sign that morphine-treated animals had exited the acute withdrawal period.

Animals next underwent a traumatic stressor in which they received 10 unsignaled footshocks. During the trauma session, morphine-treated animals displayed heightened freezing relative to saline-treated animals (Figure 1C. Effect of Morphine in Trauma Groups: $F_{1,11}$=5.19, p=0.04). However, heightened fear relative to saline-treated animals had abated by the time they were placed back into the trauma context the next day for the trauma test, although animals that experienced the trauma generally showed very high levels of freezing (Figure 1D. Effect of Morphine: $F_{1,25}$=0.26, p=0.62; Morphine x Trauma Interaction: $F_{1,25}$=0.26, p=0.62; Effect of Trauma: $F_{1,25}$=141.62, p<0.001). Notably, morphine-treated animals did not display altered motor reactivity to footshock during the trauma session (t_{11}=0.68, p=0.51; Data not shown), suggesting that shock sensitivity did not drive the differences in freezing that were seen.

Animals were then placed into a novel context and given a single mild footshock. When first placed in the novel context (i.e., prior to shock), traumatized animals displayed very little generalized freezing relative to animals that had not experienced the trauma (2% vs 0.6%; Effect of Trauma: $F_{1,25}$=4.48, p=0.04), and morphine and saline-treated animals did not differ (Effect of Morphine: $F_{1,25}$=0.42, p=0.53; Morphine x Trauma Interaction: $F_{1,25}$=0.93, p=0.344; Data not shown). However, when returned to this context the day after receiving the minor stressor, morphine-treated animals displayed a robust sensitization of fear (Figure 1E): animals that experienced the trauma froze more in the context that had been paired with the minor stressor (Effect of Trauma: $F_{1,25}$=21.48, p<0.001), and morphine-treated animals displayed this enhancement following trauma to a much greater degree than saline-treated animals (Morphine x Trauma Interaction: $F_{1,25}$=7.71, p=0.01). Although morphine increased freezing amongst animals that had received the trauma (t_{11}=2.95, p=0.01), morphine-treated animals that had not experienced the trauma did not display heightened fear levels relative to saline-treated animals (t_{14}=1.46, p=0.17), reflective of an interaction between opioid treatment history and trauma history.

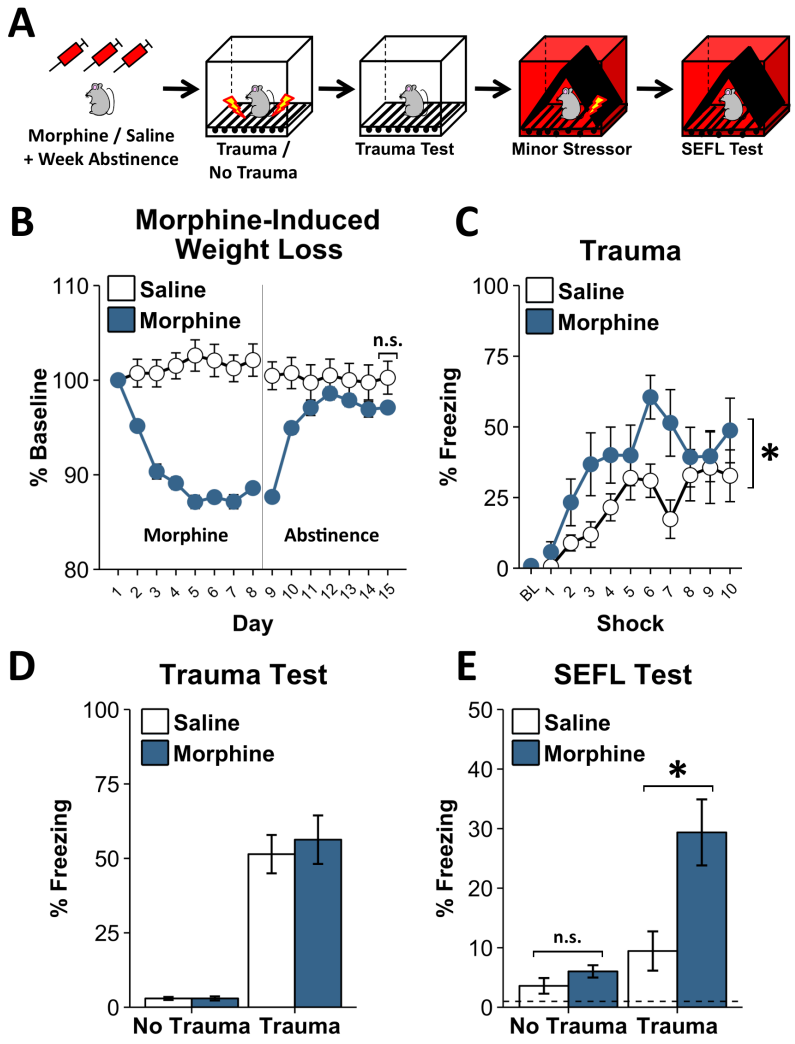

Figure 1. Experiment 1: Chronic morphine exposure and withdrawal potentiates stress-enhanced fear learning (SEFL). A) Schematic of the SEFL procedure. During the Trauma, animals receive 10, 1 mA shocks. The next day they are returned for the Trauma Test. Subsequently, animals receive a single 0.5 mA shock in a novel environment (Minor Stressor). The following day they are placed back in this environment for the SEFL Test. B) Animals were given twice daily morphine injections for 8 days, during which they lost substantial body weight. However, by the end of the weeklong abstinence period when they received the trauma, morphine-treated animals were no longer different than saline-treated animals. C) Throughout the Trauma, morphine-treated animals froze more than saline-treated animals, but D) were not different when returned for a Trauma Test the next day. E) After being given a single shock in a novel environment, animals that underwent trauma freeze more than non-trauma animals when returned to that environment (SEFL Test) – evidence of SEFL. Dashed line in E reflects average pre-shock baseline freezing of trauma animals on the previous day. Error bars reflect standard error of the mean. Asterisk reflects significance at $p<0.05$. n.s. = not significant. BL = Baseline.

Experiment 2: Chronic morphine exposure and withdrawal increases SEFL independent of pain sensitivity and anxiety

The finding that animals treated chronically with morphine display enhancements in SEFL could potentially be explained by a state of hyperallodynia, a common consequence of chronic opioid treatment, or by a general state of anxiety induced by acute withdrawal. However, we did not find evidence to support either of these claims (Figure 2). Instead, it is likely that differences in SEFL are a consequence of fundamentally altering the processes that support fear learning.

A separate set of animals was treated using the escalating morphine regimen described above, and after a week of abstinence, these animals were tested in the EPM (Figure 2A). Morphine and saline-treated animals did not differ with respect to the percentage of time they spent exploring the open arms of the EPM, suggesting that chronic morphine exposure did not produce a persistent state of anxiety (Figure 2B; t_{22}=1.23, p=0.23). A subset of these animals was then assessed for their reactivity to a range of shock amplitudes, ranging from 0.1 mA (barely detectable), to 0.5 mA, the amplitude used for the minor stressor. Although shock-induced motion increased monotonically in accordance with shock amplitude (Figure 2C. Effect of Intensity: $F_{8,80}$=13.14, p<0.001), morphine-treated animals did not differ from salin- treated animals (Effect of Morphine: $F_{1,10}$=3.86, p=0.08; Morphine x Intensity interaction: $F_{8,80}$=1.13, p=0.36). This finding indicates that reactivity to shock cannot explain differences in SEFL in morphine-treated animals.

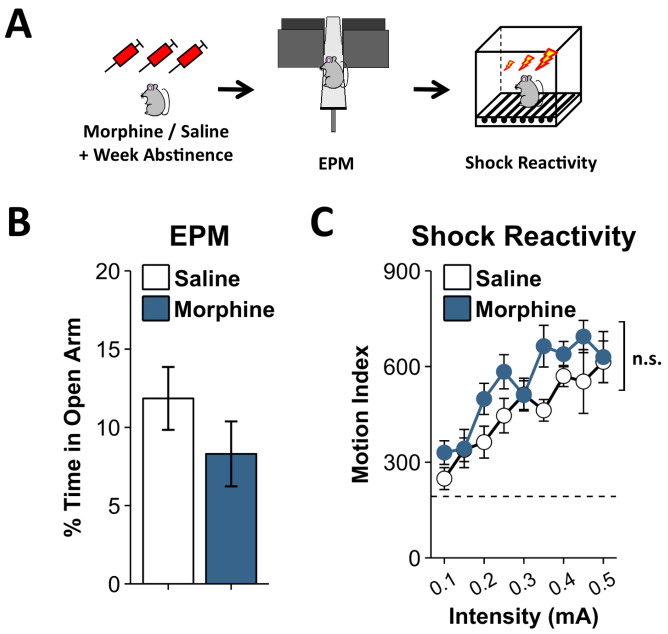

Figure 2. Experiment 2: Chronic morphine exposure and withdrawal increases SEFL independent of pain sensitivity and anxiety. A) Experiment Schematic. A week after chronic morphine/saline exposure, animals were tested in the EPM. The next day, shock reactivity was assessed. B) Morphine-treated animals did not display altered exploration of the open arms of the EPM. C) Morphine-treated animals did not display altered shock reactivity. Dashed line in C reflects pre-shock motion prior to the first shock, which did not differ between groups (t_{10}=0.95, p=0.36). Error bars reflect standard error of the mean. n.s. = not significant.

Experiment 3: Frequency of morphine administration and withdrawal does not alter morphine-induced enhancements in SEFL

The ability of morphine to induce enhancements in SEFL could be a consequence of morphine exposure, *per se*, or the extent to which morphine exposure induces physiological dependence and withdrawal. In Experiment 3, we manipulated the frequency of morphine administration, without altering the cumulative amount of morphine received, in an effort to begin disentangling these possibilities. This experiment revealed that despite dramatically altering morphine's ability to induce long-term physiological changes – reflected in reduced weight loss and reduced time being on drug per day – altering the frequency of morphine administration did not impact morphine's ability to enhance SEFL (Figure 3).

Animals were treated with morphine/saline either twice or once daily, receiving a total of 16 injections over 8 or 16 days, respectively. A week after the last injection, they were run through the SEFL procedure (Figure 3A). Notably, mice treated with morphine once per day lost substantially less weight than mice treated twice per day (Figure 3B; Group x Day Interaction: $F_{14,175}=17.04$, $p<0.001$), giving credence to the notion that these mice were substantially less affected by morphine treatment. By the end of morphine administration, animals treated with morphine once per day had lost weight relative to saline-treated animals (Figure 3B. Injection 15 weight difference: $t_{18}=3.95$, $p<0.001$), but animals treated with morphine twice per day had lost even more weight than those treated with morphine once per day (Figure 3B. Injection 15 weight difference: $t_{14}=4.28$, $p<0.001$).

A week after the last morphine injection, animals were run through the SEFL procedure. Regardless of the frequency of morphine administration, morphine-treated animals showed an enhancement in both the trauma memory (Figure 3C. Effect of Morphine: $F_{1,24}=9.7$, $p<0.01$; Effect of Frequency: $F_{1,24}=1.66$, $p=0.21$; Morphine x Frequency Interaction: $F_{1,24}=0.04$, $p=0.85$),

and SEFL (Figure 3D. Effect of Morphine: $F_{1,24}$=12.68, p<0.01; Effect of Frequency: $F_{1,24}$=1.18, p=0.29; Morphine x Frequency Interaction: $F_{1,24}$=0.59, p=0.45).

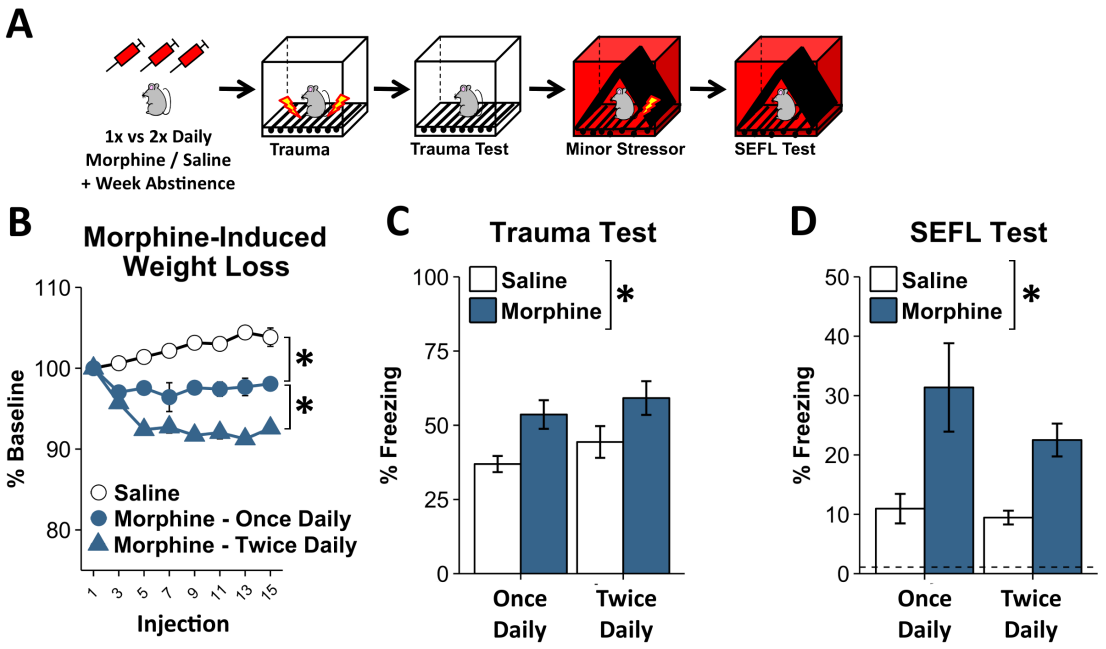

Figure 3. Experiment 3: Frequency of morphine administration and withdrawal does not alter morphine-induced enhancements in SEFL. A) Animals were given 16 saline/morphine injections, either over 8 or 16 days, and a week later were run through the SEFL procedure. B) Twice-daily morphine produces more robust weight loss than once-daily morphine. C) Irrespective of injection frequency, morphine treatment potentiated the trauma memory, D) and SEFL. Dashed line in D reflects average pre-shock baseline freezing of trauma animals on the previous day. Error bars reflect standard error of the mean. Asterisk reflects significance at $p<0.05$. Asterisks next to legend denote main effect of morphine treatment.

Experiment 4: Preventing morphine withdrawal does not mitigate morphine-induced enhancements in SEFL

To provide an even more drastic manipulation of withdrawal, we implanted mice with subcutaneous morphine pellets (or placebo pellets), which have been shown to provide a continual release of morphine that drops off slowly over 5-7 days (Dighe, Madia, Sirohi, & Yoburn, 2009; McLane et al., 2017). This allowed us to prevent mice from cycling through repeated withdrawal. Moreover, although withdrawal cannot be ruled out entirely, the slow decay of opioid release by morphine pellets ensures a tapering off process that should attenuate withdrawal.

Then, in order to experimentally mimic the repeated withdrawal process, half of the animals received twice-daily injections of the highly selective mu opioid receptor antagonist naltrexone (0.25 mg/kg, i.p.) over the course of 7 days, which precipitates withdrawal for several hours after each injection. A week after the last naltrexone injection, all animals were run through the SEFL procedure (Figure 4A). Remarkably, morphine treatment facilitated fear learning independent of the frequency of withdrawal (Figure 4).

To confirm that we were effectively able to produce withdrawal across the course of naltrexone treatment, we examined defecation and jumping behavior – two classic measures of precipitated withdrawal – every other day across the period of naltrexone administration. As can be seen in Figure 4B, only morphine animals injected with naltrexone displayed increases in these behaviors (Defecation Statistics [Morphine x Naltrexone Interaction: $F_{1,23}=56.03$, $p<0.001$; Effect of Naltrexone in Morphine Group: $F_{1,11}=126.9$, $p<0.001$; Effect of Naltrexone in Placebo Group: $F_{1,12}=0.36$, $p=0.55$]; Only morphine-naltrexone animals showed any jumping behavior).

Moreover, we looked at weight changes across the period of naltrexone administration. Similar to morphine injections, morphine pellet implantation resulted in significant weight loss in morphine-treated animals on the day following pellet implantation, prior to the first naltrexone

injection (Figure 4C: Effect of Morphine: $F_{1,25}$=34.4, p<0.001). However, naltrexone drastically sped the rate of weight recovery across the 7 days of naltrexone treatment, as can seen in Figure 4C (Morphine x Naltrexone Interaction: $F_{1,23}$=23.58, p<0.001; Morphine x Naltrexone x Day Interaction: $F_{6,138}$=2.37, p=0.07). Although naltrexone increased the weight of animals implanted with morphine over this period (Effect of Naltrexone in Morphine Animals: $F_{1,11}$=48.53, p<0.001), it had no impact in placebo animals (Effect of Naltrexone in Placebo Animals: $F_{1,12}$=0.89, p=0.37; Naltrexone x Day Interaction: $F_{6,72}$=0.45, p=0.71). Indeed, by the final day of naltrexone treatment, morphine mice injected with naltrexone weighed significantly more than the other three groups of animals, which did not themselves differ (Contrast of Morphine-Naltrexone vs Other Groups: $F_{1,25}$=21.92, p<0.001; no differences between the other three groups: $F_{2,17}$=0.33, p=0.73).

Despite the enormous differences in withdrawal behaviors and weight and withdrawal exhibited between groups, freezing was similarly enhanced in morphine-exposed groups. Both in the trauma test and the final SEFL test, animals implanted with morphine pellets expressed enhanced freezing relative to animals implanted with placebo pellets, and there was no impact of naltrexone treatment (Figures 4D-E. Trauma Test Statistics [Effect of Morphine: $F_{1,23}$=5.29, p=0.03; Effect of Naltrexone: $F_{1,23}$=1.61, p=0.22; Morphine x Naltrexone Interaction: $F_{1,23}$=2.56, p=0.12]. SEFL Test Statistics [Effect of Morphine: $F_{1,23}$=5.13, p=0.03; Effect of Naltrexone: $F_{1,23}$=1.56, p=0.22; Morphine x Naltrexone Interaction: $F_{1,23}$=0.45, p=0.83]). Consequently, morphine withdrawal is unlikely to have produced the effects we observed on fear learning.

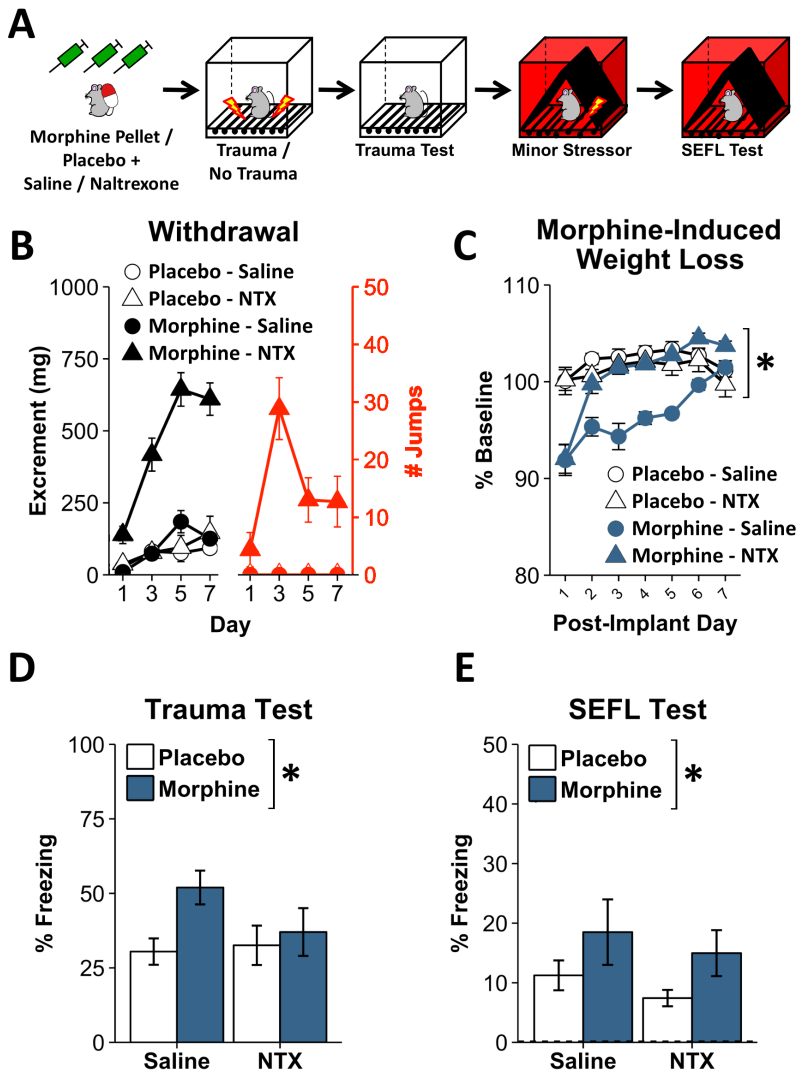

Figure 4. Experiment 4: Blocking morphine withdrawal does not mitigate morphine-induced enhancements in SEFL. A) Experiment Schematic. Animals were implanted with placebo/morphine pellets, provided a sustained release of morphine, and were then given twice daily injections of saline/naltrexone twice/day for 7 days to precipitate withdrawal. A week after the last saline/naltrexone injection, all animals were run through the SEFL procedure. B) Morphine animals treated with naltrexone display robust withdrawal. C) Morphine-induced weight loss is overcome by naltrexone-precipitated withdrawal. D) Irrespective of the amount of withdrawal, animals implanted with morphine pellets display heightened fear in the Trauma Test, E) and the SEFL Test. Dashed line in E reflects average pre-shock baseline freezing on the previous day. Error bars reflect standard error of the mean. Asterisk reflects significance at $p<0.05$. Asterisks next to legend denote main effect of morphine treatment.

Experiment 5: Chronic morphine given after traumatic experience weakly enhances fear learning

Theories regarding the relationship between PTSD and drug dependence often posit that drug use emerges in an effort to self-medicate (i.e. drug use follows trauma), and epidemiological evidence supports the idea that PTSD diagnosis increases risk for SUD and drug use (Chilcoat & Breslau, 1998a, 1998b). In order to better model this temporal relationship, we next assessed the impact of morphine exposure on enhanced fear learning when morphine was administered after initial trauma but before the minor stressor (Figure 5). In addition to addressing how opioid use subsequent to trauma might influence stress sensitization and the progression of PTSD, because this was the first time a fear memory acquired prior to morphine administration was examined subsequent to it, this experiment allowed us to further assess whether morphine acted merely to impact *fear expression*, or whether it altered *fear learning*.

Animals were given the same traumatic experience described previously, and the following day began the 8-day regimen of chronic morphine followed by one week of abstinence before being tested in the rest of the SEFL procedure (Figure 5A). Upon being returned to the trauma context, animals given morphine did not display altered fear of the trauma context relative to saline-treated animals (Figure 5B. Effect of Morphine: $F_{1,46}$=0.16, p=0.69; Morphine x Trauma Interaction: $F_{1,46}$=0.11, p=0.74). Thus, morphine experience did not alter expression of a previously acquired fear memory.

Then, when animals were given a mild aversive foot-shock in a novel context, morphine-treated animals displayed a trend towards increased freezing in this context the following day, but this did not reach significance (Figure 5C. Effect of Morphine: $F_{1,46}$=2.87, p=0.1; Effect of Trauma: $F_{1,46}$=27.12, p<0.001; Morphine x Trauma Interaction: $F_{1,46}$=0.12, p=0.73). The finding that giving morphine after trauma reduces the ability of morphine to augment SEFL supports the notion that morphine potentiates the ability of subsequent trauma to enhance fear learning.

Moreover, the fact that morphine did not alter the expression of a previously learned fear memory suggests that morphine acts directly to alter fear learning, rather than the expression of fear.

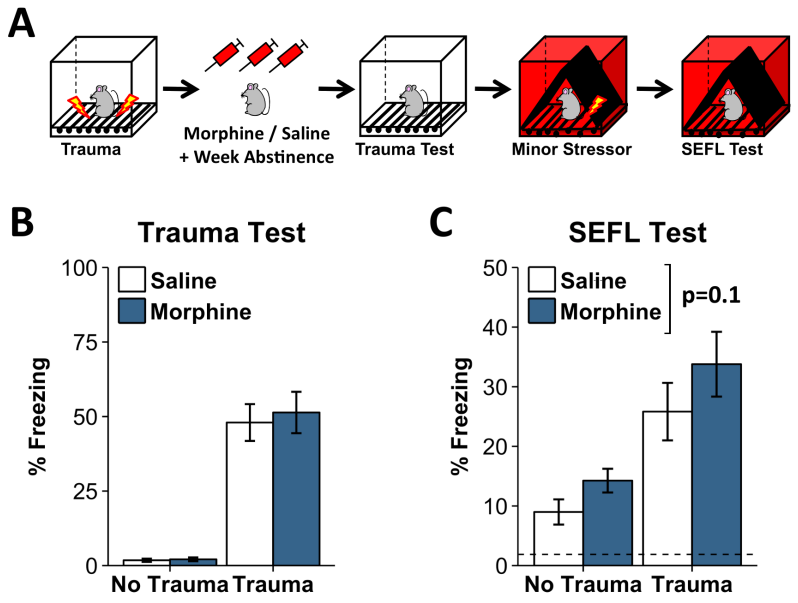

Figure 5. Experiment 5: Chronic morphine given after traumatic experience weakly enhances fear learning. A) Experiment Schematic. Chronic morphine and a week of abstinence were given in-between the Trauma and the rest of the SEFL procedure. B) Morphine-treated animals do not display altered fear of the trauma context when placed back into it. C) Morphine-treated animals display a trend toward heightened fear in the SEFL test, but this did not reach significance. Dashed line in C reflects average pre-shock baseline freezing on the previous day. Error bars reflect standard error of the mean.

Experiment 6: Potentiation of fear learning by chronic opioid treatment decays with time

In order to address whether the sensitization of SEFL following morphine exposure was permanent, it was next examined whether the same morphine regimen would increase fear learning if animals were run through the SEFL procedure a month after discontinuation of morphine (Figure 6A). Morphine-treated animals did not display heightened fear of the trauma context when tested at this point (Figure 6B. t_{14}=0.18, p=0.86). Additionally, when placed back into the context paired with the mild footshock, morphine- and saline-treated animals that had experienced trauma did not differ (Figure 6C. t_{14}=0.15,p=0.89). Thus, although the sensitization of SEFL lasts beyond the period of acute withdrawal, it does not persist indefinitely.

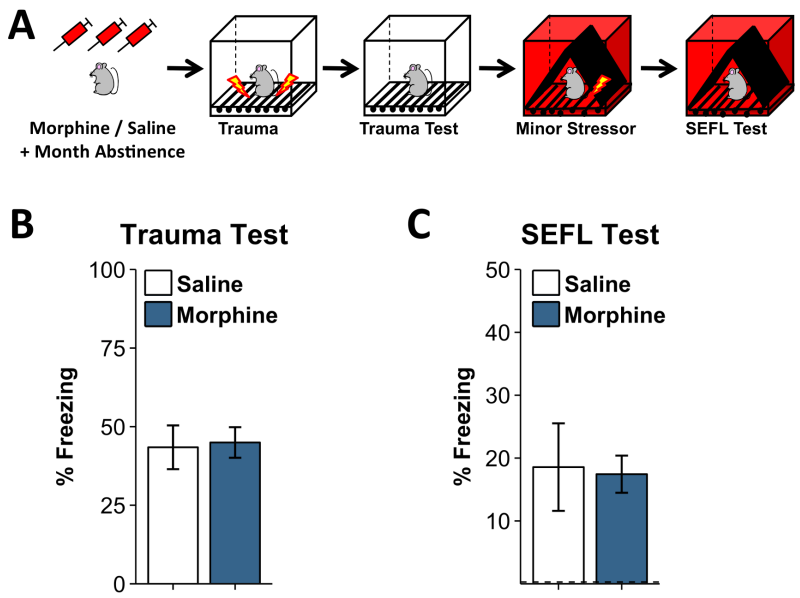

Figure 6. Experiment 6: Potentiation of fear learning by chronic opioid treatment decays with time. A) Experiment Schematic. Animals underwent the SEFL procedure a month after chronic saline/morphine administration. B) At this time-point, morphine-treated animals do not display altered fear of the trauma context, C) nor do they show augmented SEFL. Dashed line in C reflects average pre-shock baseline freezing on the previous day. Error bars reflect standard error of the mean.

Experiment 7: Regulation of immediate early genes by chronic morphine exposure

Having determined that chronic morphine administration is able to robustly alter fear learning, without altering shock sensitivity, anxiety, or the expression of fear, we next sought to identify potential regions of interest upon which morphine might act to mediate these effects (Figure 7). We focused on immediate early gene expression within key nodes of the fear circuit that also express opioid receptors: the BLA, CEA, and dorsal BNST (Le Merrer et al., 2009).

Animals were treated chronically with morphine, and a week later were either exposed to the EPM to induce c-Fos expression, or remained in their home cage, and tissue was subsequently taken for immunohistochemistry (Figure 7A). Notably, we used the EPM to induce c-Fos expression because we were interested in activity that predates differences in fear learning, and at the same time needed a task that we knew would engage anxiety/fear circuitry.

Although c-Fos was induced in the BLA and CEA in response to EPM exposure (Figure 7B-D. BLA: $t_{12}=3.61$, $p<0.01$; CEA: $t_{12}=2.14$, $p=0.05$; BNST: $F_{12}=1.49$, $p=0.16$), there were no differences in EPM-induced c-Fos between saline- and morphine-treated animals in these regions (BLA: $t_{12}=0.19$, $p=0.85$; CEA: $t_{12}=0.68$, $p=0.57$).

Additionally, because GluA1 expression was previously found to be increased in the BLA of animals that had undergone the SEFL trauma (Perusini et al., 2016), we also looked at BLA GluA1 expression levels. Here as well, no differences were found in morphine-treated animals (Figure 7E. $t_9=0.16$, $p=0.88$). Although future studies using direct electrophysiological assessments are in order, these studies suggest that morphine does not exert its influence merely by altering the number of cells engaged in the fear circuit.

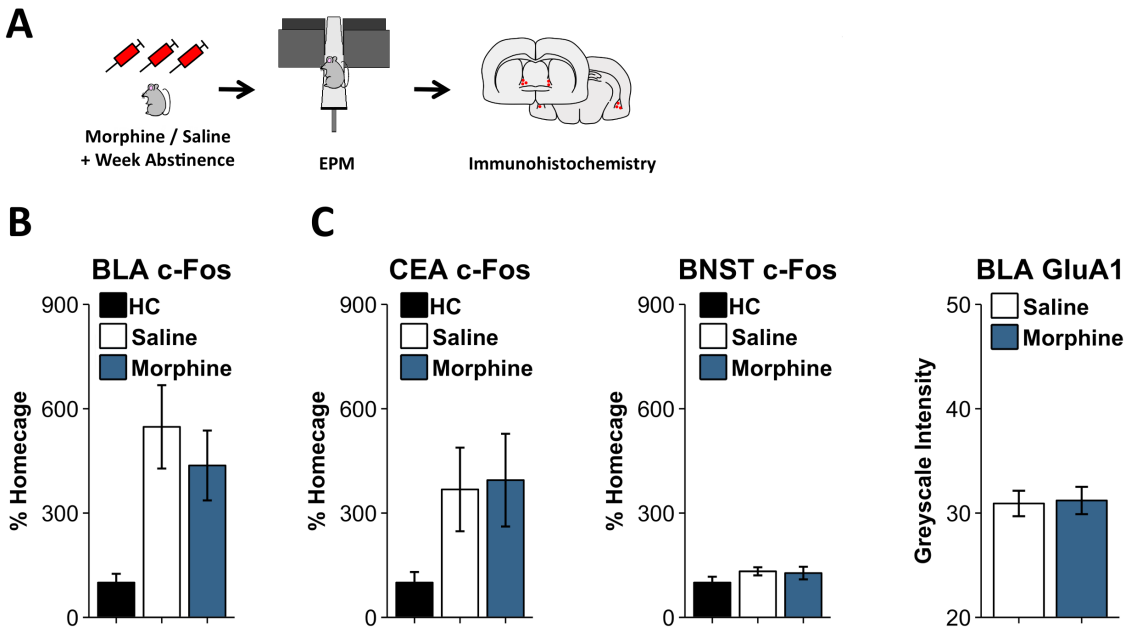

Figure 7. Experiment 7: Regulation of immediate early genes by chronic morphine exposure. A) Experiment Schematic. Animals received the chronic morphine regimen, or saline, and after a week of abstinence were tested in the EPM prior to taking tissue for immunohistochemistry. B-D) Although EPM induced c-Fos in the BLA and CEA, morphine did not cause differential activation of these regions. E) Additionally, GluA1 receptor expression was not different in the BLA of morphine-treated animals. Error bars reflect standard error of the mean.

Experiment 8: Post-learning increase in BLA excitability in morphine-treated animals

In Experiment 7, analysis of immediate early gene activation across the fear circuitry failed to identify regional differences in morphine- and saline-treated animals prior to fear conditioning. As a consequence, we next examined immediate early gene activity and GluA1 levels in the BLA of morphine- and saline-treated animals after the final SEFL test (Figure 8A). The BLA was focused on because it is thought to be the central locus of plasticity supporting fear learning (M. S. Fanselow & Gale, 2003; M. S. Fanselow & LeDoux, 1999).

Although the SEFL Test induced c-Fos in the BLA (t_{13}=3.47, p<0.01), there were no differences between saline- and morphine-treated animals (Figure 8B. t_{13}=1.5, p=0.16). In contrast, changes in GluA1 in the BLA had a remarkable ability to predict morphine-induced enhancements in SEFL. GluA1 levels were heightened in the BLA of morphine-treated animals (Figure 8C. t_9=2.55,p=0.03). To further explore this relationship, we next examined the relationship between freezing in the final SEFL test and GluA1 levels in a large set of animals. Multiple regression was performed to jointly predict SEFL test freezing from GluA1 levels and morphine treatment (y=B0+B1*GluA1+B2*Morphine+e). This revealed that GluA1 was a significant predictor of freezing (Figure 8D. B1=6.36, t=2.56, p=0.02), above and beyond the influence of morphine (B2=9.67, t=1.99, p=0.06). Impressively, the overall model was capable of predicting 50% of the variance in freezing ($F_{2,20}$=9.86, p=0.001, R=0.7, R^2=0.5). These findings suggest that although morphine may not enhance SEFL by altering the number of recruited BLA cells, it may instead alter their excitability.

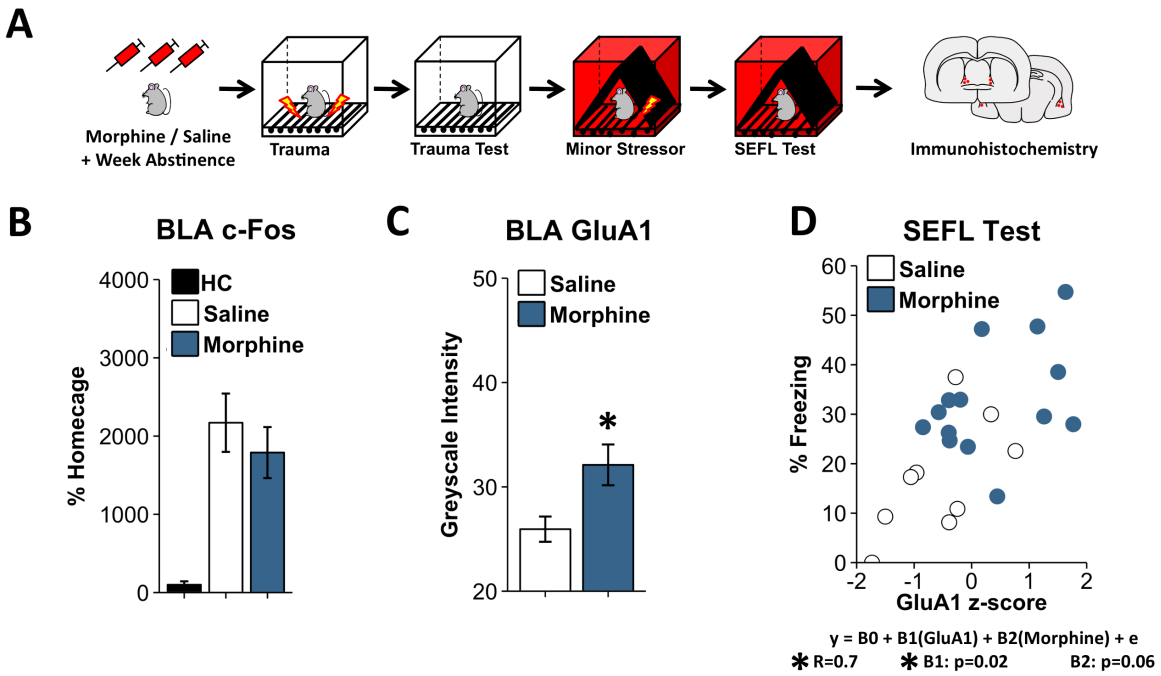

Figure 8. Experiment 8: Post-learning increase in BLA excitability in morphine-treated animals. A) Experiment Schematic. Animals received the chronic morphine regimen, or saline, and after a week of abstinence underwent the SEFL procedure. Tissue for immunohistochemistry was taken after the final SEFL test. B) Although the SEFL Test induced c-Fos in the BLA, saline- and morphine-treated animals did not differ. C) Subsequent to the SEFL Test, morphine-treated animals had greater levels of GluA1 in the BLA relative to controls, D) and GluA1 levels predict final SEFL Test freezing, controlling for morphine treatment. Multiple regression model used to predict SEFL Test freezing is presented at bottom. Error bars reflect standard error of the mean. Asterisk reflects significance at $p<0.05$.

Section 2:

Contributions of Kappa Signaling to Morphine-Induced Enhancements in SEFL

Experiment 9: Kappa receptor antagonism fails to reduce morphine-induced enhancements in SEFL

The release of the endogenous opioid dynorphin and its actions upon kappa opioid receptors has been suspected to contribute to withdrawal-induced anxiety, propelling subsequent drug use (Chavkin & Koob, 2016; Schlosburg et al., 2013). Moreover, kappa receptor antagonists have been explored for their potential as anxiolytics with low abuse liability (Knoll et al., 2007). In order to assess whether dynorphin release contributes to the impact of opioids on SEFL, and moreover, whether kappa receptor blockade might be targeted to reduce opioid-induced anxiety, we administered the long-acting kappa receptor antagonist JDTic throughout the period of morphine administration, abstinence, and SEFL (Figure 9A). Counter to the *a priori* hypothesis that JDTic would mitigate morphine-induced enhancements in SEFL, it was of no consequence, irrespective of morphine exposure.

To first confirm that the dose of JDTic to be used was able to produce lasting antagonism of the kappa opioid receptor, we demonstrated that it could block kappa agonist-induced hypolocomotion days after initial administration. Animals were injected with either saline or JDTic (10 mg/kg, i.p.) and three days later their baseline locomotion was assessed; it did not differ (Figure 9B. t_{10}=0.236, p=0.82). Then, over the next two days, animals' locomotor response to a high dose of the kappa agonist U-50488H (10 mg/kg, i.p.), and then saline, was examined (Figure 9B). JDTic-treated animals showed a significant attenuation in U50-induced hypolocomotion, without showing altered locomotion following a saline injection (Figure 9B. JDTic x Drug Interaction: $F_{1,10}$=6.217, p=0.03; U50 response: t_{10}=2.61, p=0.03; Saline response: t_{10}=0.09, p=0.93).

Having confirmed that JDTic can produce lasting antagonism of kappa receptors, in a separate set of animals we then examined the ability of JDTic to mitigate morphine-induced increases in SEFL. During morphine administration, JDTic-treated animals lost less weight in

response to chronic morphine exposure, suggesting that blocking dynorphin release was able to alter the impact of morphine on the animals (Figure 9C). This was evidenced by an interaction between morphine exposure and JDTic treatment across the 8 days morphine was injected (Morphine x JDTic Interaction: $F_{1,83}$=4.85, p=0.03). Whereas morphine-treated animals differed in response to JDTic (Effect of JDTic: $F_{1,45}$=19.625, p<0.001; JDTic by Day: $F_{7,315}$=5.483, p<0.001), saline-treated animals did not (Effect of JDTic: $F_{1,38}$=3.38, p=0.07; JDTic by Day: $F_{7,266}$=1.34, p=0.25).

As done previously, after a week of abstinence animals were run through the SEFL protocol. Although morphine exposure again enhanced SEFL, JDTic did not attenuate this enhancement, nor did it reduce signs of fear in morphine-naïve animals (Figures 9D-E). JDTic had no impact on trauma session freezing (Effect of JDTic: $F_{1,83}$=0.03, p=0.868; JDTic x Trial Interaction: $F_{9,747}$=0.36, p=0.93; JDTic x Morphine Interaction: $F_{1,83}$=1.44, p=0.23; JDTic x Morphine x Trial Interaction: $F_{9,747}$=1.95, p=0.06), nor did it alter the strength of the trauma memory when animals were returned to the trauma context the next day (Figure 9D. Effect of JDTic: $F_{1,83}$=0.43, p=0.84; JDTic x Morphine Interaction: $F_{1,83}$=0.05, p=0.82). Furthermore, JDTic had no impact on the ability of morphine to potentiate the SEFL phenotype when animals that had previously experienced trauma were placed back into a context paired with a mild aversive stimulus (Figure 9E: Effect of JDTic: $F_{1,83}$=0.9, p=0.35; JDTic x Morphine Interaction: $F_{1,83}$=0.6, p=0.44). Nevertheless, morphine was again able to potently increase freezing during this final test (Effect of Morphine: $F_{1,83}$=17.39, p<0.001).

It is notable that JDTic did have a significant impact on generalized fear when morphine-treated animals were initially placed into the context of the mild aversive stimulus, before its delivery (JDTic x Morphine Interaction: $F_{1,87}$=4.17, p=0.04). Here, morphine-treated animals that had not been treated with JDTic generalized relative to saline-treated animals (t_{41} =2.42, p=0.02), but morphine-treated animals treated with JDTic did not show this increase in fear

generalization (t_{42}=0.141, p=0.89). This was the only sign of reduced fear seen in JDTic-treated animals and was modest at best, as even morphine-treated animals not treated with JDTic only froze at 3.6% during this period.

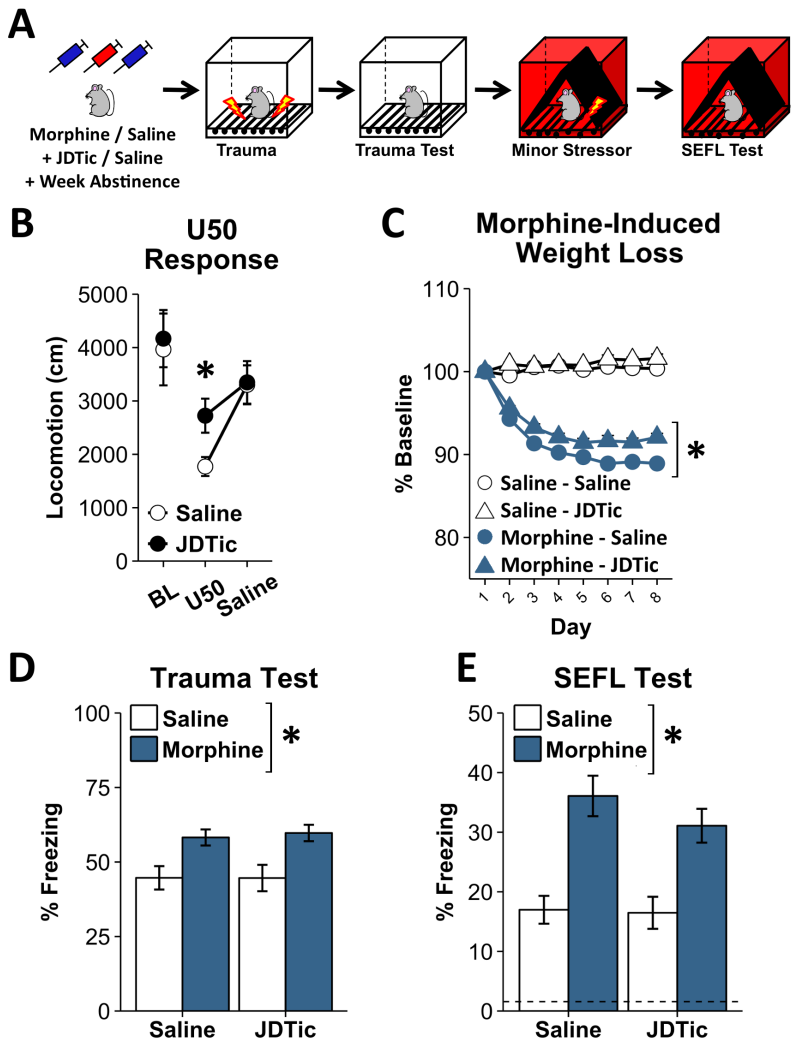

Figure 9. Experiment 9: Kappa receptor antagonism fails to reduce morphine-induced enhancements in SEFL. A) Experiment Schematic. Animals received the chronic morphine regimen, or saline, and after a week of abstinence underwent the SEFL procedure. Saline or JDTic was administered every 3-4 days throughout morphine administration, abstinence, and SEFL, in order to provide continual antagonism of kappa receptors. B) In a separate subset of animals, it was shown that the dose of JDTic used is able to attenuate kappa agonist-induced hypolocomotion at least 4 days after a single JDTic injection. C) JDTic reduces weight loss resulting from chronic morphine administration. D) JDTic does not alter the ability of morphine to augment fear in the Trauma Test, E) nor did it mitigate morphine's potentiation of SEFL. Dashed line in E reflects average pre-shock baseline freezing on the previous day. Error bars reflect standard error of the mean. Asterisk reflects significance at $p<0.05$. BL = Baseline.

Experiment 10: Kappa receptor antagonism fails to reduce anxiety and depression-like behavior

Because several reports have suggested that kappa receptor antagonism reduces signs of anxiety/depression and drug-seeking, we were very surprised to find that JDTic did not reduce fear and anxiety in the SEFL model. Thinking that our failure to find an effect might be influenced by the nature of the test, we also assessed the impact of JDTic on two classic models of anxiety and depression which have both been shown to be sensitive to manipulations of dynorphin/kappa (Knoll et al., 2007; McLaughlin et al., 2003): the EPM and a 2-day version of the forced swim test (Figure 10). Nevertheless, JDTic again failed to reduce signs of anxiety and depression. Animals were administered JDTic/saline and two days later were assessed in the EPM, followed by the forced swim test. In the EPM, JDTic treated animals did not spend a greater percentage of time in the open arms, indicating that they were not less anxious (Figure 10B. t_{13} =1.08, p=0.3). In the forced swim test, which began the day after the EPM, JDTic treated animals also did not differ with respect to immobility during the first day of exposure (Figure 10C. t_{14} =0.45, p=0.66), nor did they demonstrate differences in immobility across four consecutive test sessions on the following day (Figure 10C. Effect of JDTic: $F_{1,14}$=0.15, p=0.7; JDTic x Session Interaction: $F_{1,14}$=0.4, p=0.54), the second day previously being found to be more sensitive to dynorphin deletion (McLaughlin et al., 2003). Thus, despite having clear evidence that the dose administered in this experiment was sufficient to block kappa receptors and could influence morphine-induced weight loss, kappa receptor antagonism did not alter measures of fear, anxiety and depression in our hands.

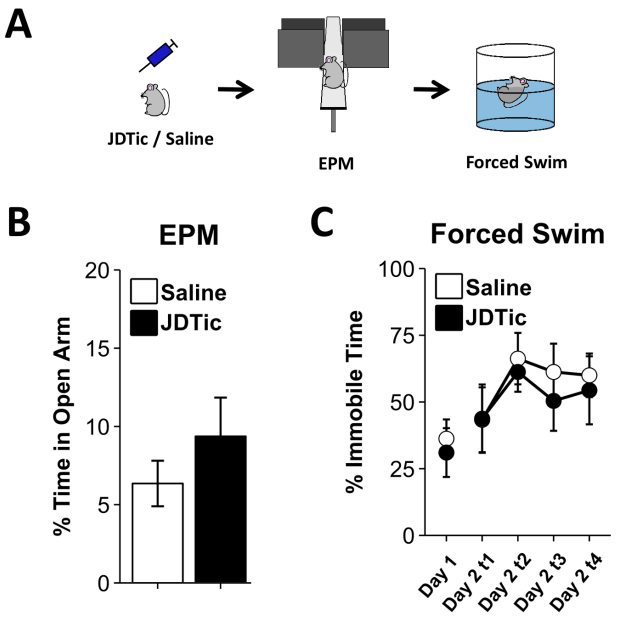

Figure 10. Experiment 10: Kappa receptor antagonism fails to reduce anxiety and depression-like behavior. A) Experiment Schematic. Animals received JDTic/saline and 2 days later were tested in the EPM. Across the next two days, they were tested in the forced swim test, utilizing a procedure previously demonstrated to be sensitive to dynorphin deletion and kappa antagonism. B) JDTic did not alter time spent in the open arms of the EPM. C) JDTic did not alter time immobile in the forced swim test, either on the first day or across 4 exposures (t1-t4) on the second day. Error bars reflect standard error of the mean. Asterisk reflects significance at $p<0.05$.

Section 3:

Potentiation of Opioid Sensitivity by Prior Traumatic Experience

Experiment 11: Prior traumatic experience potentiates opioid sensitivity

Having provided an extensive characterization of how a prior history of opioid exposure alters SEFL, I also sought to understand how traumatic experience influences drug-associated behaviors. In particular, although it is known that the SEFL trauma is able to enhance BLA-dependent aversive learning, it is less clear whether it is able to enhance BLA-dependent appetitive learning. To this end, mice were administered the SEFL trauma or were given equivalent contextual exposure without being shocked, and a week later, were trained and tested for morphine CPP: an associative learning procedure that is dependent upon the BLA (Figure 11A). In this experiment it was found that trauma robustly potentiated locomotor responses to morphine (20 mg/kg, i.p.), a sign that drug sensitivity is enhanced in animals that have experienced trauma, but did not affect preference for the morphine-paired environment. Across the 4 training sessions, morphine-treated animals showed a robust increase in their locomotor response to morphine (Figure 11B. Morphine x Trauma Interaction: $F_{1,13}=12.5$, $p<0.01$; Effect of Trauma on Morphine Locomotion: $F_{1,13}=12.79$, $p<0.01$; Effect of Trauma on Saline Locomotion: $F_{1,13}=0.05$, $p=0.82$). However, when tested in a drug-free state, as well as in the presence of morphine, trauma exposed animals did not display altered preference for the morphine-paired side of the CPP chamber (Figure 12C. Effect of Trauma: $F_{1,13}=0.05$, $p=0.82$; Trauma by Test Interaction: $F_{1,13}=0.69$, $p=0.42$). Thus, although trauma was able to alter opioid responses, this did not appear to result in changes in associative learning about opioids.

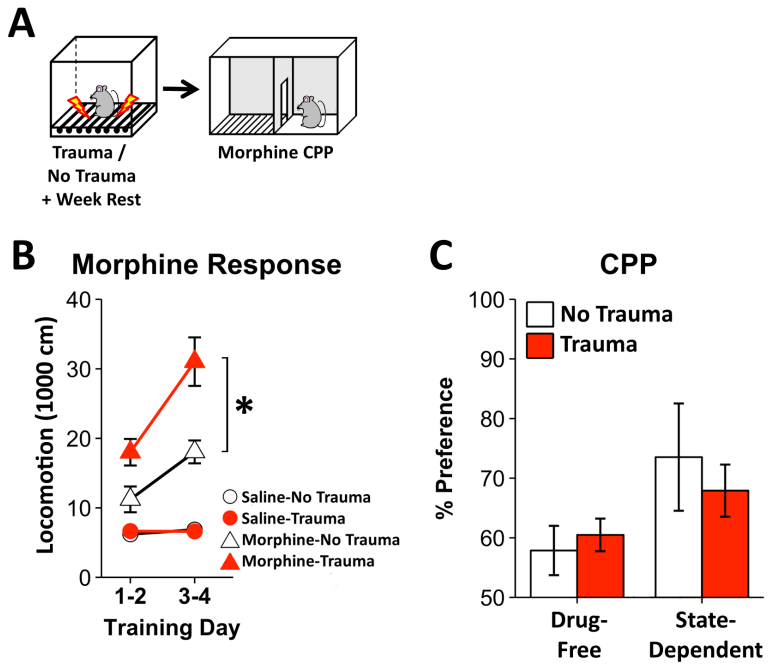

Figure 11. Experiment 11: Prior traumatic experience potentiates opioid sensitivity. A) Experiment Schematic. Animals underwent the SEFL Trauma or received equivalent contextual exposure without being shocked. A week later, animals learnt to associate one side of a CPP chamber with morphine during 4 training sessions (2 saline, 2 morphine). B) Trauma-exposed animals displayed a robust augmentation of the locomotor response to morphine during training. C) However, trauma and no trauma animals displayed a similar preference for the morphine-paired compartment during both drug-free and state-dependent CPP test sessions. Error bars reflect standard error of the mean. Asterisk reflects significance at $p<0.05$.

Discussion

Opioids Potentiate the Ability of Trauma to Augment Fear Learning

The experiments in the first section of this dissertation highlight a dramatic and previously undocumented ability of chronic opioid exposure to potentiate stress-enhanced fear learning, a finding that may provide insight into the comorbidity between PTSD and opioid use and dependence. Given that this potentiation lasts for some time, though not indefinitely, beyond discontinuation of drug exposure, it is possible that sensitization of fear learning predisposes individuals who have used opioids – either as prescribed or illicitly – to PTSD.

It is important to emphasize that the observed enhancement in fear appears to be a consequence of a facilitation of the biological processes that give rise to the formation of fear memories, as opposed to some other cognitive or physiological process that influences fear, such as stimulus aversiveness or anxiety.

First, great care was taken to insure that the differences observed in morphine-treated animals were not the consequence of increased sensitivity to shock, the aversive stimulus used in these experiments. Animals treated with morphine did not differ in their locomotor response to a wide range of shock amplitudes, precluding the possibility that differences in fear resulted from mere differences in shock sensitivity. Although it could be argued that these motor responses do not parallel the internal subjective response following shock, this is unlikely to be the case. After repeated trials, maximal freezing to a stimulus paired with an aversive event is directly related to the magnitude of that aversive event (Morrow, Elsworth, Rasmusson, & Roth, 1999; Young & Fanselow, 1992). However, in Experiment 1, we found that despite freezing during the trauma being higher in morphine-treated animals (i.e., during learning), when animals were returned to this environment freezing was not different, further suggesting that the aversiveness of the stimulus was not different. Although in some experiments morphine-treated animals froze more during the trauma test, average freezing during the trauma test for saline-

treated animals in these experiments was lower than in Experiment 1 (Experiment 1: 51%; Experiment 3: 40%; Experiment 4: 30%). Therefore, it is possible that learning may not have been complete in the latter experiments. Moreover, it is imperative to note that much of the behavioral and subjective responses that occur even shortly after shock are entirely dependent upon learning. For example, freezing after shock has been demonstrated not to be an unconditional/reflexive response to shock, but is instead a reflection of conditional/learnt response to the environment in which the shock was experienced (Fanselow, 1980; Fanselow, 1986). As such, the immediate motor responses to shock are the best indicators of stimulus sensitivity.

The increase in fear learning observed is also unlikely to be a consequence of a general state of increased anxiety. Morphine-treated animals did not differ in their exploration of the open arms of an EPM at a time in which they show heightened fear learning, suggesting that they were not more anxious. Moreover, when animals experienced a trauma prior to morphine exposure, animals did not differ with respect to freezing when subsequently returned to the context of the traumatic event, providing evidence that expression of a previously learned fear response is unaffected. Consequently, in addition to these data showing that morphine did not alter sensitivity to the aversive stimulus used, morphine-treated animals were also not generally more anxious or likely to express a previously acquired fear response. Instead, morphine appears to have altered the ability with which negative experiences affect future behavior – that is to say, learning.

These experiments also demonstrate that morphine treatment interacts with subsequent trauma to potentiate fear learning, as morphine-treated animals showed minimal evidence of increased fear learning in the absence of a later traumatic event. In Experiment 1, morphine-treated animals that did not receive trauma did not display heightened fear learning, whereas those that experienced trauma very much did. Moreover, when morphine treatment followed a

traumatic event, enhancements in fear learning were small and did not vary based upon prior trauma exposure. These findings suggest that although morphine exposure increases fear learning, it has an even more dramatic effect on the extent to which traumatic stressors are able to sensitize fear-learning systems.

In conjunction with the finding that morphine exposure enhances SEFL, altered markers of synaptic plasticity within the BLA were also found in morphine-treated animals. Chronic morphine treatment resulted in post-learning increases in the GluA1 subunit of the AMPA receptor within the BLA, but GluA1 levels in the BLA prior to fear conditioning were not different between saline- and morphine-treated animals. Moreover, the degree of GluA1 increase was found to be highly predictive of the amount of stress-enhanced fear learning displayed. Importantly, the BLA is thought to be the site of synaptic plasticity supporting associative fear learning (Duvarci & Pare, 2014; M. S. Fanselow & LeDoux, 1999), production and insertion of GluA1 AMPA receptors are a correlate of long-term potentiation (LTP) (Makino & Malinow, 2009), and blocking incorporation of these receptors into the membrane within the amygdala has been shown to block associative fear learning (Rumpel et al., 2005). Thus, in addition to the behavioral indicators that fear learning is enhanced, we also find that the critical molecular cascades that support fear learning are similarly enhanced.

Despite finding evidence of post-learning correlates of enhanced SEFL in morphine-treated animals, immediate early gene expression failed to reveal tentative latent predictors of enhanced fear learning across several nuclei thought to be robust nodes within the fear circuitry. Although such methods are commonly employed as a preliminary means of extracting regional differences in ‘excitability’ or ‘activation,’ failure of such methods to discriminate cell type, as well determining degree of activation, are known limitations. Indeed, it is striking that despite displaying massive differences in freezing responses and BLA GluA1 expression after the final SEFL test, morphine- and saline-treated animals showed no differences in BLA c-Fos activation.

Perhaps immediate early gene methods are better at detecting which populations of cells are engaged at a given time point as opposed to how active a population is. Nevertheless, because markers of synaptic plasticity within the BLA were augmented by morphine, future research will hopefully identify intrinsic changes in the BLA, or in BLA afferents, following morphine exposure.

Perhaps the most striking of findings from the first section is that repeated and robust withdrawal did not augment the ability of morphine exposure to enhance SEFL. Moreover, reducing the frequency of morphine administration so that it was less disruptive (i.e., animals were on drug for a shorter period each day and lost less weight) similarly did not mitigate enhancements in SEFL. These findings indicate that the enhancement in SEFL by morphine is not a consequence of the stress of repeated morphine withdrawal, but instead might be a consequence of cumulative morphine exposure. It is worth mentioning that despite manipulating physical withdrawal, these experiments did not directly compare the amount of physiological dependence induced by various morphine procedures. It is conceivable that the critical variable is whether or not physiological dependence is produced, irrespective of withdrawal. Regardless, because physiological dependence is often unavoidable in the medicinal use of opioids, even when withdrawal can be avoided, the finding that changing the amount of physical withdrawal experienced does not change the potentiation seen is of great clinical interest.

In closing, these results provide compelling evidence that chronic opioid exposure is able to robustly sensitize stress-enhanced fear learning. Given the striking comorbidity between PTSD and opioid dependence, as well as the growing prevalence of opioid use and dependence in our society, these findings further caution their safety.

Kappa Antagonism Fails to Alter Anxiety and Depression

In Section 2, antagonism of the kappa opioid receptor via the long-acting antagonist JDTic was found to be unable to reduce the enhancements in SEFL produced by chronic opioid administration. Moreover, JDTic had no ability to alter freezing behavior in saline-treated animals, nor was it able to alter measures of anxiety or depression in the EPM and forced swim tasks, respectively. Given a sizable body of literature indicating that kappa antagonists are anxiolytic (Bruchas et al., 2010; Knoll et al., 2007; Land et al., 2008; McLaughlin et al., 2006; McLaughlin et al., 2003), and that kappa antagonism is able to reduce stress-mediated enhancements in drug-seeking (Chavkin & Koob, 2016; Schlosburg et al., 2013), these findings are surprising.

It could be argued that the dose of JDTic utilized was insufficient. However, we found that the dose of JDTic used was able to substantially attenuate locomotor suppression produced by a high dose of the kappa receptor agonist U50 – which has previously been shown to support aversive conditioning (Land et al., 2008) – for several days following injection. Moreover, because JDTic was administered several times to morphine-treated animals, cumulative dosing insures even greater antagonism. Lastly, this dose of JDTic was previously shown to produce anxiolytic effects (Knoll et al., 2007). Therefore, these results are unlikely to be attributable to a less than substantial antagonism of kappa receptors. Instead, it appears endogenous dynorphin release does not regulate these behaviors, at least under the experimental conditions employed here.

It should be noted that we did observe some behavioral and physiological responses to JDTic in morphine-treated animals. First, we found that JDTic was able to reduce the amount of morphine-induced weight loss seen during chronic morphine administration. Whether or not this effect was mediated by actions on feeding, metabolism or activity; and moreover, whether this effect is centrally or peripherally mediated, is unclear. It is interesting that prior studies suggest that activation of kappa receptors increases feeding, whereas their inhibition reduces feeding

(Kavaliers, Teskey, & Hirst, 1985; Levine, Grace, Billington, & Portoghese, 1990; Walker, Katz, & Akil, 1980). However, because we did not observe weight changes in morphine-naïve mice, it is likely that the changes seen interact with some specific effect of morphine exposure that alters weight, as opposed to having a general impact on feeding.

In addition, we found that morphine-treated mice showed evidence of greater generalized fear following trauma, and that this was reduced by treatment with JDTic. Because even morphine-treated animals showed very low levels of generalized freezing (~3%), these findings are difficult to interpret. Nevertheless, it may be the case that although kappa signaling has no impact on the acquisition of a fear memory, or stress-enhanced fear learning, it may indeed influence the extent to which fear memories generalize. Future studies that are more directly suited to probing this question may identify such a circumscribed role for dynorphin in the modulation of fear.

To a lesser extent, it may alternatively be the case that various kappa receptor antagonists differentially regulate intracellular signaling pathways leading to kappa-mediated increases in fear and anxiety. nor-BNI and JDTic, two of the most frequently used kappa receptor antagonists, both work in a somewhat non-traditional manner, wherein transient binding to the kappa receptor produces antagonism of intracellular signaling lasting several weeks, despite no longer occupying the receptor (Bruchas et al., 2007). Perhaps there are differences in the intracellular pathways altered by these antagonists. However, despite nor-BNI having been more frequently used in prior studies of anxiety (Beardsley et al., 2005; McLaughlin et al., 2006; McLaughlin et al., 2003; Schlosburg et al., 2013), JDTic has previously been shown to alter both unconditional and conditional measures of fear and anxiety (Chartoff et al., 2012; Knoll et al., 2007). Thus, the notion that the drugs act on distinct molecular pathways seems unlikely.

This is not the first report of discrepant effects of kappa antagonism on affective behaviors. nor-BNI was found to produce antidepressant-like effects in the forced swim test in Wistar Kyoto, but not Sprague-Dawley rats, despite desipramine being effective in both strains (Carr et al., 2010). Moreover, direct infusion of U50 into the nucleus accumbens versus the lateral septum was reported to produce bi-directional effects on measures of anxiety (Wang et al., 2016). Lastly, although direct deletion of kappa receptors from dopamine neurons is able to attenuate anhedonia produced by chronic social defeat stress, JDTic was ineffective; moreover, neither manipulation was able to reduce social avoidance following chronic social defeat stress (Donahue et al., 2015). These results suggest that individual variation in ligand/receptor expression across brain regions, or state differences, may predict the efficacy of kappa antagonism in altering depression/anxiety behaviors. Moreover, such efficacy may be restricted to specific cognitive/behavioral endpoints related to anxiety/depression.

In sum, our results, in conjunction with prior discrepant findings on the effects of kappa antagonism, suggest a nominal and nuanced role for dynorphin release in anxiety and depressive-like behavior. Moreover, in light of recent reports of cardiac concerns for the use of JDTic in humans (Buda, Carroll, Kosten, Swearingen, & Walters, 2015), these findings further call into question the clinical benefits of kappa receptor antagonists. More pre-clinical research evaluating the precise circumstances under which stress causes dynorphin release will hopefully shed better light on the clinical conditions more likely to be benefitted by kappa receptor antagonism.

Trauma Sensitizes Opioid Sensitivity but not Place Preference

In the final section, the ability of prior trauma to produce a lasting enhancement in BLA-dependent appetitive learning was assessed. Here, it was found that trauma did not alter morphine CPP, though it did produce robust changes in the ability of trauma to influence

locomotor responses to morphine. These findings are revelatory in that they suggest that SEFL is specific to BLA-dependent aversive learning, and also suggest a path forward for studying how trauma influences drug responses.

In discussing these results, it is important to remember the motivations for performing this work: first, to identify a procedure that would allow for subsequent studies addressing how trauma-mediated brain changes confer individuals to addiction; and second, to assess whether the SEFL trauma selectively potentiates learning about aversive stimuli.

With respect to the first goal, this work is promising in that it suggests that the SEFL trauma is able to produce lasting changes in responses to opioids. Moreover, although this change in behavior does not directly capture changes in drug preference or drug-seeking (which of course was the goal), drug-induced locomotor responses have been found to be predictive of drug-taking as well as other traits related to addiction. For example, in a set of mouse strains segregated based upon levels of impulsivity in a reversal learning task in which food was the reinforcer, it was found that impulsive strains of mice showed greater locomotor responses to cocaine, faster acquisition of an instrumental response for cocaine, and maintained higher asymptotic rates of instrumental responding for cocaine (Cervantes, Laughlin, & Jentsch, 2013). Moreover, utilizing a different acute stress procedure, it was demonstrated that prior stress experience in rats is able to potentiate CPP for low-dose morphine and oxycodone, but not non-opioid drug classes, and locomotor sensitivity to these drugs tracked the CPP changes observed (Amat et al., 2005; Der-Avakian et al., 2007; Will et al., 2002; Will, Watkins, & Maier, 1998). Therefore, the observed differences in locomotor responses to morphine in trauma-exposed animals may be supported by changes that confer increases in other addiction-related phenotypes. Ongoing work seeks to address this question.

It is intriguing that the prior work demonstrating that stress is able to induce increased opioid-CPP and accompanying changes in opioid sensitivity also recruited the use of repeated

shock. Because shock leads to analgesia, it would be interesting to see if repeated shock procedures lead to lasting changes in the tone and sensitivity of endogenous opioids. Findings of decreased shock reactivity in SEFL trauma animals (Poulos et al., 2014) may suggest higher basal opioid tone despite increased aversive learning. Moreover, it is possible that by altering opioid tone, physical traumas could influence sensitivity to opioids, providing a possible mechanism for the link between opioid use/dependence and PTSD.

With respect to the second goal of this work, the results suggest that the SEFL trauma does not potentiate BLA-dependent appetitive learning. Although it could be argued that there is something procedural that prevented us from detecting an increase in CPP, this is unlikely. Firstly, we performed a minimal number of morphine pairings relative to the bulk of CPP studies. Moreover, our drug-free test showed relatively low levels of CPP, suggesting that an enhancement was not out of range. Lastly, the fact that prior work has shown that stress sensitizes opioid CPP but not CPP for other drug classes (Der-Avakian et al., 2007; Will et al., 1998), strongly suggests that a general potentiation of the appetitive learning processes of the BLA are not altered by trauma, as this would presumably persist across multiple drug classes. Given the reported intermingling of neurons representing positive and negative stimuli within the BLA (Beyeler et al., 2016; Gore et al., 2015), this suggests that there is a mechanism for selectively enhancing plasticity in BLA-pathways that support aversive learning in response to trauma. Given that there are methods currently available for long-term activity-dependent tagging of different cell types, future work might demonstrate molecular or electrophysiological signatures of SEFL selectively in cells that code aversive stimuli.

Closing Remarks

In closing, the experiments contained in this dissertation provide mechanistic insight into the interplay between opioid use/dependence and PTSD, and moreover, call into the question

the potential efficacy of kappa antagonists for the treatment drug addiction and anxiety/depression. It is demonstrated that a prior history of opioid exposure is capable of augmenting the acquisition of fear memories, a critical process underlying PTSD development. Moreover, it is shown that traumatic experience is able to augment opioid sensitivity, which could in turn influence the development of opioid dependence. Lastly, it is found that kappa receptor antagonism does little to influence opioid-induced changes in fear learning, and was not able to alter measures of anxiety and depression. These findings have important clinical implications for the treatment of SUDs, PTSD and other affective disorders, as well as pain conditions in which opioids are used for treatment.

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