Published in final edited form as:

Neuroscience. 2017 December 16; 366: 1–14. doi:10.1016/j.neuroscience.2017.09.055.

Neurobiological Correlates of Pain Avoidance-Like Behavior in Morphine-Dependent and Non-Dependent Rats

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Abstract

Repeated use of opioids can lead to the development of analgesic tolerance and dependence. Additionally, chronic opioid exposure can cause a paradoxical emergence of heightened pain sensitivity to noxious stimuli, termed hyperalgesia, which may drive continued or escalated use of opioids to manage worsening pain symptoms. Opioid-induced hyperalgesia has traditionally been measured in rodents via reflex-based assays, including the von Frey method. To better model the cognitive/motivational dimension of pain in a state of opioid dependence and withdrawal, we employed a recently developed non-reflex-based method for measuring pain avoidance-like behavior in animals (mechanical conflict avoidance test). Adult male Wistar rats were administered an escalating dose regimen of morphine (opioid-dependent group) or repeated saline (control group). Morphine-dependent rats exhibited significantly greater avoidance of noxious stimuli during withdrawal. We next investigated individual relationships between pain avoidance-like behavior and alterations in protein phosphorylation in central motivation-related brain areas. We discovered that pain avoidance-like behavior was significantly correlated with alterations in phosphorylation status of protein kinases (ERK, CaMKII), transcription factors (CREB), presynaptic markers of neurotransmitter release (Synapsin), and the rate-limiting enzyme for dopamine synthesis (TH) across specific brain regions. Our findings suggest that alterations in phosphorylation events in specific brain centers may support cognitive/motivational responses to avoid pain.

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Author Disclosure Statement: The authors declare no competing financial interests or potential conflicts of interest.

Keywords

Opioid Dependence; Avoidance; Pain; Prefrontal Cortex; Striatum; Hippocampus

INTRODUCTION

Opioid addiction (or opioid use disorder) is a chronic, relapsing psychiatric disease associated with the emergence of negative affective states, that parallels an escalation of opioid use over time (Koob and Le Moal, 1997; Edwards and Koob, 2013). In select vulnerable individuals, the transition from recreational opioid use to addiction is thought to involve a shift from positive to negative reinforcement processes underlying the motivation for opioid use (Edwards and Koob, 2010). In such cases, opioids may be sought after and taken in excessive amounts to alleviate withdrawal-related affective symptoms (Massaly et al., 2016). Opioid analgesics such as morphine are also widely used for the treatment of severe or chronic pain (Fields, 2011), acting primarily via mu-opioid receptors within nociceptive circuitry (Fields, 2004). However, repeated or excessive use of opioids often leads to the development of analgesic tolerance (DeLander et al., 1984; Aceto et al., 1986). Moreover, a well-documented side effect of chronic opioid use is the paradoxical emergence of heightened pain sensitivity to noxious stimuli, termed hyperalgesia (Angst and Clark, 2006). Enhanced pain sensitivity can even play a role in cue-induced opioid craving during attempted abstinence (see Ren et al., 2009), reflecting the highly interactive nature of nociception and opioid addiction mechanisms. To better understand this phenomenon, opioid-induced hyperalgesia has been traditionally modeled at the preclinical level in rodents experiencing withdrawal from chronic opioid administration via reflex-based assays of nociceptive sensitivity (e.g., McNally and Akil, 2002; Edwards et al., 2012).

Pain is a multidimensional construct. In addition to somatosensory elements, both affective and cognitive/motivational dimensions exist and contribute to pain-related morbidity (Egli et al., 2012). Chronic pain in particular can engender a sustained negative affective state and a reorganization of cognitive strategies to avoid pain, while relief from pain is rewarding (Becerra and Borsook, 2008; Porreca and Navratilova, 2017). It is thus hypothesized that the emergence of painful states following chronic or excessive opioid exposure facilitates negative reinforcement processes whereby individuals seek relief from pain by escalating their opioid use, culminating in the development of psychiatric sequelae including opioid use disorder (Shurman et al., 2010; LeBlanc et al., 2015). Recent efforts toward understanding these processes have established operant methods to measure pain-related behaviors in rodents (e.g., King et al., 2009), with the hope of providing additional construct and translational validity with regard to the interaction of pain and motivational (or goal-directed) behavior.

At the neurobiological level, mu-opioid receptors in the ventral striatum (Olds, 1982), ventral tegmental area (Phillips & LePiane, 1980), and hippocampus (Stevens et al., 1991) mediate the acute rewarding effects of opioids. The ventral striatum is a brain region associated with reward, reinforcement learning, and motivation, which along with regions including the hippocampus and prefrontal cortex, has been implicated in the progression

from initial drug use to addiction (Edwards and Koob, 2010). Both drugs of abuse (Di Chiara and Imperato, 1988) and natural rewards (Fibiger et al., 1992; Pfaus, 1999; Kelley, 2004) increase dopamine neurotransmission between the ventral tegmental area and ventral striatum. In contrast, withdrawal from most abused drugs including opioids is associated with decreases in extracellular dopamine concentrations (Rossetti et al., 1992), and this event is closely linked to the dysphoric or negative affective symptoms of withdrawal and dependence (Volkow et al., 2002). In addition to mediating the acute rewarding effects of drugs of abuse, the ventral striatum represents a functional terminus for ascending nociceptive pathways (Gear and Levine, 1995; Chang et al., 2014) in close association with additional areas involved in the affective processing of pain, including the prefrontal cortex (Wei & Zhuo, 2001; Vogt, 2005) and hippocampus (Ploghaus et al., 2001; Borrás et al., 2004). With time, chronic pain states can produce functional abnormalities in the prefrontal cortex (Apkarian et al., 2004; Metz et al., 2009), hippocampus (Mutso et al., 2012; Vachon-Preseau et al., 2013), and striatum (Baliki et al., 2012). As in addicted states, dopamine deficits also manifest during persistent pain (Hipólito et al., 2015; Taylor et al., 2016; Massaly et al., 2016) in close association with negative affective symptomatology and pain chronification (Jarcho et al., 2012; Tiemann et al., 2014; Borsook et al., 2016).

The present study was designed to investigate the motivation to avoid pain in morphine-dependent and non-dependent animals, and to measure associations between pain avoidance and alterations in protein phosphorylation in motivation-related brain regions. We employed a novel and non-reflex-based method for measuring the motivation to avoid noxious stimuli during withdrawal from chronic morphine administration, termed the mechanical conflict avoidance test (Harte et al., 2016). We tested the hypothesis that morphine-dependent animals would be more motivated to avoid noxious mechanical stimuli during opioid withdrawal. We also measured phosphoprotein-level neuroadaptations in cognitive/motivation-related brain regions associated with pain avoidance-like behavior across both morphine-dependent and non-dependent groups to better understand the neurobiological correlates of this behavior.

EXPERIMENTAL PROCEDURES

Animals

Adult male Wistar rats were purchased from Charles River and weighed 200-300 grams at the time of arrival. Rats were pair-housed and given *ad libitum* access to food (Purina Rat Chow, Ralston Purina, St. Louis, MO) and water throughout behavioral training and testing. Rats were maintained on a reverse 12-hour light/dark cycle (lights off at 8:00 am). Rats were handled regularly and given one week to acclimate to the colony room prior to the start of experimental procedures. A total of 21 animals were used in this study. Behavioral testing was completed in two separate cohorts (group 1: $n = 5$ chronic morphine-injected, $n = 5$ chronic saline-injected; group 2: $n = 5$ chronic morphine-injected, $n = 6$ chronic saline-injected). All animal care, use, and procedures in this study were approved by the Institutional Animal Care and Use Committee of Louisiana State University Health Sciences Center (LSUHSC) and were in accordance with the National Institute of Health guidelines.

Mechanical Conflict-Avoidance Task

Apparatus—The mechanical conflict-avoidance task (Harte et al., 2016) is a non-reflex-based method that was employed to measure increases in the motivation to avoid noxious stimuli during withdrawal from chronic morphine administration. The mechanical conflict-avoidance apparatus (87.3 cm × 21 cm × 43.2, Noldus) contained three red acrylic chambers: a brightly lit start chamber, a probe chamber, and a dark goal chamber (Figure 1). The start chamber contained an aversive and intense LED light (wavelength ~490nm-690nm) that minimized the amount of heat that it emits. The nociceptive probe chamber was located between the start box and the goal box and contained a perforated floor with stainless steel probes that could be adjusted to different heights from the surface of the floor (0 mm, 0.5 mm, 1 mm, 2 mm, 3 mm, 4 mm, 5 mm). The tips of the probes were sharp enough to confer a noxious stimulus without causing injury to the animals (Harte et al., 2016). The probes were positioned evenly within 1 cm in any direction so that animals had to traverse over the probes to reach the dark goal chamber. The three chambers were separated by two acrylic guillotine doors that could be raised and secured by magnets to allow movement through the different chambers.

Habituation—All rats were habituated to the maze two days prior to training. Rats were placed in the start chamber with the light turned off for 10 seconds and the guillotine door closed. The light was turned on for the duration of 20 seconds after which the guillotine door that is located between the start chamber and probe chamber (0 mm) was quickly opened. Rats were allowed to freely explore between all three chambers for 5 minutes. This procedure was repeated the following day to determine if the rats displayed a clear preference for the dark chamber.

Training—Training took place over three days. Rats were placed in the start chamber (no light) for 10 seconds and the light was turned on for 20 seconds. After 20 seconds of light exposure, the guillotine door was opened and the first timer was started. The amount of time that it took for the rat to place all four paws on the probes (latency to exit onto the probes, max latency = 30 seconds) was measured. If the rat did not exit onto the probes after 30 seconds, then the animal was removed from the maze and returned to its home cage. The second timer was started once the rat exited onto the probes and stopped once the animal entered the goal chamber (latency to enter the dark chamber, max latency = 60 seconds). If the rat did not enter the goal chamber after 60 seconds, then the animal was removed from the maze and returned to its home cage. This procedure was completed four times per day with a minimum of 10 minutes between each group of a trial.

Testing—The stimulus-response assessment of mechanical conflict-avoidance involved measuring the latency to exit onto the probes at differing probes heights (0 mm, 0.5 mm, 1 mm, 2 mm, 3 mm, 4 mm, 5 mm). The procedure for each trial was identical to those previously described during training. Measurements without probes (0 mm) were taken on the first day of testing and on the first trial of every day. Each probe height was tested on a separate day following the 0 mm test and presented in the following order: Day 2: 0.5 mm, Day 3: 2 mm, Day 4: 4 mm, Day 5: 3 mm, Day 6: 1 mm, Day 7: 5 mm. Rats were separated into two groups (experimental and control) based on baseline measurement of latency to exit

onto the probes at differing probe heights. To assess mechanical conflict avoidance in morphine-dependent rats compared to saline controls, the stimulus-response assessment was repeated after two weeks of repeated daily injections (Figure 4). Stimulus-response measurements were taken each day over 7 days and the presentation of probes was the same as described above. Animals received morphine or saline injections immediately following behavioral testing each day, such that morphine-dependent animals were in acute withdrawal during the presentation of each probe height.

Von Frey Testing

Von Frey tests of mechanical hypersensitivity were conducted (Figure 3) as previously described (Edwards et al., 2012). Rats were placed in individual plastic compartments with stainless steel mesh floors for 30 minutes until the rats' grooming and exploratory behaviors ceased. To assess the presence of mechanical hypersensitivity, the mid-plantar area of each hind paw was perpendicularly stimulated with calibrated nylon Von Frey filaments for 3 seconds using the up-down method. A brisk withdrawal of the paw (often followed by a sustained retraction and/or licking, possibly indicative of supraspinal organization) is considered a positive response, but paw withdrawals due to locomotion or weight shifting were not counted. For quantitative assessment, the 50% probability withdrawal threshold, or paw withdrawal threshold, was calculated as previously described (Chaplan et al., 1994). Paw withdrawal thresholds were measured approximately 24 h following the previous morphine or saline injection.

Drugs

Morphine (Sigma Aldrich, St. Louis, MO) was dissolved in sterile saline (9% NaCl) at a concentration of 10 mg/kg or 20 mg/kg. 10 mg/kg (1 mL/kg) of morphine dissolved in sterile saline was injected subcutaneously for one week daily. 20 mg/kg (2 mL/kg) of morphine dissolved in sterile saline was injected subcutaneously for the remainder of the experiment in the same rats each day to mimic an escalating dose regimen.

Western Blot Analysis

Western blot analyses for brain regional changes in protein phosphorylation were conducted as previously described (McGinn et al., 2016) in animals from group 1. Rats were sacrificed by decapitation under light isoflurane anesthesia 24 hours after the last injection (Figure 2A). The brains were rapidly removed, snap-frozen in isopentane, and stored at -80°C until dissection. During the dissection, the brains were mounted and sliced using a cryostat. Regional brain punches (0.5 mm thick) were taken from frozen tissue using a 13-16 gauge needle according to (Paxinos and Watson, 1998) (Figure 2B–D). Brain punches were homogenized by sonication in a lysis buffer (320 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mM EDTA, 1 % SDS, protease inhibitor cocktail (diluted 1:100), and phosphatase inhibitor cocktails II and III (diluted 1:100); Sigma, St. Louis, MO, USA). Tissue homogenates were heated at 100°C for 5 minutes and stored at -80°C until the total protein concentration was measured using a detergent-compatible Lowry method (Bio-Rad, Hercules, CA, USA). Samples of protein (20 μg) were separated by SDS-polyacrylamide gel electrophoresis on 8% acrylamide gels using a Tris/Glycine/SDS buffer system (Bio-Rad). The gels were electrophoretically transferred to polyvinylidene difluoride membranes (GE Healthcare,

Piscataway, NJ, USA). Membranes were blocked for 1 hour in 5% non-fat milk at room temperature and incubated overnight in 2.5% non-fat milk with primary antibody at 4°C. Primary antibodies included phospho TH₄₀ (1:500-1:100000; EMD Millipore; Cat # AB5935), phospho CaMKII (1:50000; Cell Signaling; Cat # D21E4), phospho CREB (1:20000; EMD Millipore; Cat # 06-519), phospho Synapsin (1:2500; Cell Signaling; Cat # 2311), and phospho ERK (1:40000; EMD Millipore; Cat # 05-797R). Membranes were washed and incubated with species-specific peroxidase-conjugated secondary antibody (1:10000; Bio-Rad) for 1 hour at room temperature. Membranes were washed and incubated in a chemiluminescent reagent (SuperSignal West Pico; Thermo Scientific, Rockford, IL, USA), and exposed to film. Following film development, membranes were stripped for 30 minutes at room temperature (Restore; Thermo Scientific) and reprobed for total TH (1:10000-1:500000; EMD Millipore; Cat # MAB5280), total CaMKII (1:50000; Cell Signaling; Cat # 11945), total CREB (1:20000; EMD Millipore; Cat # 06-863), total Synapsin (1:2500; Cell Signaling; Cat # 2312), and total ERK (1:40000; Cell Signaling; Cat # 9102) levels. The immunoreactivity of the bands was detected using densitometry (Image J 1.45S; Bethesda, MD). To normalize the data across the blots the densitized values were expressed as a percentage of the mean of the chronic saline-injected controls for each gel.

Statistical Analysis

All data were analyzed using Prism 6 (GraphPad Software, Inc; La Jolla, CA). One-way repeated measures ANOVA was used to measure the effect of probe height on latency to exit onto the probes and latency to enter the dark chamber during baseline (pre-injection). After splitting animals into experimental and control groups, mechanical conflict-avoidance task data were analyzed using two-way between-subjects ANOVA with latency to exit onto the probes and probe height as factors or latency to enter the dark chamber and probe height as factors. Pearson's *r* correlations and linear regressions were used to analyze the relationships between mean latency to exit onto the probes (average of all the probe heights) and individual protein phosphorylation levels or changes in mechanical hypersensitivity. A *t*-test was used to measure the difference in latency to exit onto the probes between groups at a probe height of 0mm and to test the change in mechanical hypersensitivity in both groups. Significance levels for statistical tests was set at *p*<0.05.

RESULTS

Pain Avoidance and Mechanical Hypersensitivity in Morphine-Dependent and Non-Dependent Animals

In a mechanical conflict-avoidance task, training consisted of rats being given a choice between remaining in a lighted box (an aversive condition) or crossing over elevated probes (noxious stimulus) to reach a goal box. This procedure was completed four times per day with a minimum of 10 minutes between each group of a trial before beginning the baseline stimulus-response assessment of mechanical conflict-avoidance. Baseline assessment of mechanical conflict-avoidance involved measuring the latency to exit onto the probes and the latency to enter the dark chamber of all rats at differing probes heights (0 mm, 0.5 mm, 1 mm, 2 mm, 3 mm, 4 mm, 5 mm). During the baseline stimulus-response assessment of mechanical conflict-avoidance, we found that there was a significant effect of probe (group 1

[$F(9,54)=9.500$, $p=0.0005$]; group 2 [$F(10,60)=5.349$, $p=0.0221$]) for latency to exit onto the probes, but there was no main effect of probe (group 1 [$F(9,54)=0.8786$, $p=0.4746$]; group 2 [$F(10,60)=1.635$, $p=0.1870$]) for latency to enter the dark chamber (data not shown). This indicates that the latency to exit onto the probes was a better stimulus-response measure of pain avoidance than latency to enter the dark chamber in our animals. Based on baseline measurements of latency to exit onto the probes, rats from groups 1 and 2 were balanced into equivalent treatment groups where there was a significant main effect of probe height (group 1 [$F(6,56)=4.700$, $p=0.0006$]; group 2 [$F(6,63)=23.30$, $p=0.0077$]), but no main effect of group (group 1 [$F(1,56)=0.0448$, $p=0.8330$]; group 2 [$F(1,63)=0.1068$, $p=0.7661$]) (data not shown).

Rats were treated over three weeks with either morphine (10-20 mg/kg, to induce and maintain a state of opioid dependence) or saline injections every 24 hours. Similar morphine regimens reliably induce analgesic and other forms of tolerance, in addition to central sensitization (e.g., Enrico et al., 1997; Walker et al., 1997; Craft et al., 1999; Haugan et al., 2008; Ferrini et al., 2017). To determine if the escalating morphine regimen (10-20 mg/kg) increased mechanical hypersensitivity during morphine withdrawal, we measured paw withdrawal thresholds (von Frey) at the end of weeks 1 & 2 of treatment. In morphine-dependent animals in withdrawal, there was a significant decrease in von Frey thresholds between weeks 1 and 2 in both group 1 [$t(1,8)=2.351$, $p=0.0466$] (Figure 3A) and group 2 [$t(1,6)=3.743$, $p=0.0057$] (Figure 3B), indicating heightened mechanical hypersensitivity. In contrast, in saline-treated controls there was no difference in von Frey thresholds between weeks 1 and 2 in either group 1 [$t(1,8)=0.1093$, $p=0.9157$] (Figure 3C) or group 2 [$t(1,6)=0.4525$, $p=0.6606$] (Figure 3D). To assess mechanical conflict-avoidance in morphine-dependent rats compared to saline controls, the stimulus-response assessment was repeated after this two-week period of repeated daily injections.

Mechanical conflict-avoidance testing for each day when morphine-dependent rats were in acute 24-hour withdrawal. For mechanical conflict-avoidance testing, there was a significant main effect of probe height in both group 1 [$F(6,56)=11.51$, $p<0.0001$] (Figure 4A) and group 2 [$F(6,63)=8.721$, $p<0.0001$] (Figure 4B), indicative of the noxious nature of increasing probe heights. We also found a significant main effect of treatment in group 1 [$F(1,56)=5.498$, $p=0.0226$] (Figure 4A) and group 2 [$F(1,7)=21.75$, $p<0.0001$] (Figure 4B) for latency to exit onto the probes. However, there were no interactive effects in either group 1 [$F(6,56)=0.7803$, $p=0.5889$] (Figure 4A) or group 2 [$F(6,63)=1.123$, $p=0.3591$] (Figure 4B). After combining groups 1 & 2, we found a significant main effect of probe height [$F(6,133)=16.22$, $p<0.0001$] and a significant main effect of group [$F(1,133)=18.76$, $p<0.0001$] for latency to exit onto the probes, but again no interaction effect for these factors [$F(6,133)=1.328$, $p=0.2490$] (Figure 4C). This demonstrated that morphine-dependent rats exhibit a longer latency to exit onto the probes compared to controls. Consistent with the baseline data, there was not a significant effect of probe height [$F(6,133)=1.025$, $p=0.4117$] or group [$F(1,133)=0.2315$, $p=0.6312$] for latency to enter the dark chamber (data not shown), indicating that differences in pain avoidance-like behavior was evident in the latency to exit onto the probes measurement. No group differences in performance were seen in the absence of the probes (0 mm, no noxious stimulus) [$t(19)=1.371$, $p=0.1862$], indicating that both groups exhibited similar locomotor capacity and were motivated to exit

the light chamber and reach the goal box in the absence of noxious stimuli (Figure 4C). Moreover, changes in mechanical sensitivity (Von Frey test) over the week prior to mechanical conflict avoidance testing negatively correlated with individual levels of subsequent pain avoidance-like behavior ($r=-0.4413$, $p=0.0452$; Figure 4D). These results indicate that morphine-dependent rats displayed increased mechanical hypersensitivity that at least partially overlaps with pain avoidance-like behavior during opioid withdrawal.

Neurobiological Correlates of Pain Avoidance in Reward- and Pain-Related Brain Regions

We next investigated relationships between the mean latency to exit onto the probes (measure of pain avoidance-like behavior) during the mechanical conflict-avoidance task and alterations in protein phosphorylation in and pain- and motivation-related brain regions (Table 1). We discovered levels of region-specific protein phosphorylation to be significantly correlated with pain avoidance-like behavior across groups. In the dorsomedial prefrontal cortex (dmPFC), Synapsin^{S9} phosphorylation ($r=-0.7569$, $p=0.0113$) was negatively correlated with latency to exit onto the probes (Figure 5A). Additionally, phosphorylation of cyclic AMP response-element binding protein (CREB^{S133}) in dmPFC ($r=-0.6493$, $p=0.0422$) was negatively correlated with latency to exit onto the probes (Figure 5B). These results indicate that activation of Synapsin and CREB in the dmPFC of individual animals are negatively associated with pain avoidance-like behavior. In the adjacent ventromedial prefrontal cortex (vmPFC), Synapsin^{S9} phosphorylation ($r=0.7645$, $p=0.0100$) was positively correlated with latency to exit onto the probes (Figure 6A). Phosphorylation of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII^{Thr286}) in the vmPFC ($r=0.6835$, $p=0.0293$) was also positively correlated with latency to exit onto the probes (Figure 6B). This suggests that higher pain avoidance-like behavior was associated with increased levels of pSynapsin and pCaMKII in the vmPFC.

In the ventral striatum (VS), TH^{S40} phosphorylation was positively correlated with latency to exit onto the probes ($r=0.7495$, $p=0.0126$) (Figure 7A). As tyrosine hydroxylase is activated via phosphorylation at serine 40 when there is a deficit of dopamine (Molinoff & Axelrod, 1971), this finding may indicate that differential regulation of dopamine biosynthesis is associated with pain avoidance. In comparison, CaMKII^{Thr286} phosphorylation in the VS ($r=-0.7881$, $p=0.0068$) was negatively correlated with latency to exit onto the probes (Figure 7B), suggesting that increased pain avoidance is associated with decreased autophosphorylation of CaMKII in the VS of individual animals. In contrast to the VS, in the dorsal striatum (DS), TH^{S40} phosphorylation was negatively correlated with latency to exit onto the probes ($r=-0.6774$, $p=0.0314$) (Figure 7C), indicating that altered dopamine biosynthesis in the dorsal striatum was also associated with pain avoidance behavior. Finally, phosphorylation of extracellular signal-regulated kinase (pERK) in the dorsal hippocampus (HIP) was positively correlated with latency to exit onto the probes ($r=0.7223$, $p=0.0183$) (Figure 8), suggesting that higher pain avoidance is associated with increased ERK activity in the HIP. These results demonstrate that pain avoidance-like behavior is significantly correlated with alterations in protein phosphorylation status across specific brain regions. These alterations in phosphorylation status were specific to behavioral output from the mechanical conflict-avoidance task, as von Frey thresholds did not significantly correlate with the phosphoprotein levels examined ($p>0.05$ across all substrates).

and regions, data not shown), again suggesting an imperfect overlap between these two animal models of pain sensitivity.

DISCUSSION

The present study was designed to investigate the motivation to actively avoid noxious stimuli during morphine withdrawal and to determine if pain avoidance-like behavior was associated with changes in protein phosphorylation in motivation-related brain areas. We tested the hypothesis that chronically morphine-treated rats would demonstrate increased pain avoidance-like behavior during opioid withdrawal. Morphine-dependent rats did display a significantly longer latency to exit onto the probes at 24-hours withdrawal compared to saline-treated controls, possibly reflecting increased pain avoidance in morphine-dependent rats. Additionally, our behavioral findings demonstrated that increasing the height of the probes increased pain avoidance-like behavior in both morphine-dependent rats and saline controls. We also tested relationships between pain avoidance-like behavior and alterations in protein phosphorylation in motivation-related brain regions including the dorsomedial prefrontal cortex (dmPFC), ventromedial prefrontal cortex (vmPFC), dorsal striatum (DS), ventral striatum (VS), and dorsal hippocampus (DH). We found that individual pain avoidance-like behavior was significantly correlated with brain region-specific levels of pSynapsin, pCREB, pCaMKII, pERK, and pTH in individual animals (Figure 9). Our findings suggest that alterations in these phosphorylation events may facilitate highly organized behavioral responses to avoid pain during opioid withdrawal.

There is substantial evidence that chronic overuse of opioids can lead to the paradoxical emergence of heightened pain sensitivity (or hyperalgesia) in humans (Angst and Clark, 2006) and rodents (McNally and Akil, 2002; Edwards et al., 2012). We found that morphine-dependent rats demonstrate pain avoidance-like behavior in a novel motivation-based assay (Harte et al., 2016). Traditional paw withdrawal methods such as von Frey (Chaplan et al., 1994) or Hargreaves (Hargreaves et al., 1988) represent reflex-based methods commonly used in measuring drug withdrawal-induced somatic hypersensitivity (e.g., Edwards et al., 2012; Roltsch Hellard et al., 2016). Such reflex-based pain methods determine where a stimulus reaches a noxious threshold and causes an animal to elicit a withdrawal response. The advantage of the mechanical conflict-avoidance system is that it provides a better method for modeling the cognitive and motivational dimensions of pain based on the presumed need for greater organized central behavior necessary to actively avoid noxious stimuli (Harte et al., 2016). Accordingly, continued use of this model may allow us to better understand the cognitive and motivational dimensions of chronic pain and develop more suitable treatment options.

It is important to note that we assayed pain avoidance-like behavior and neurobiological correlates during acute opioid withdrawal (i.e., instead of during continuous exposure). Our data presumably reflect unmasked neuroadaptations occurring on an intermittent basis during each subsequent withdrawal period. Withdrawal-induced changes in brain reward deficits (Kenny et al., 2006) and enhancement of opioid-seeking behavior (Lenoir and Ahmed, 2007) are manifest in opioid-dependent animals, and may correspond with altered pain sensitivity. Chronic pain causes sustained emotional distress, while relief from pain is

rewarding (King et al., 2009; Navratilova et al., 2015), and these relationships may drive the long-lasting addictive potential of opioid medications in pain-sensitive individuals (Ren et al., 2009). Our molecular findings within central motivation circuitry support previous evidence suggesting that negative affective states associated with opioid withdrawal may promote escalation of drug intake to alleviate pain (Hipólito et al., 2015; Taylor et al., 2016). We chose to investigate phosphoprotein-level neuroadaptations in motivation-related brain regions including the prefrontal cortex, striatum, and dorsal hippocampus. Dysregulation of signaling within these areas has been implicated in the transition to drug addiction (Edwards & Koob, 2010; Egli, et al., 2012; Pahng et al., 2017). Corticostriatal circuitry is also intimately involved in affective self-regulation (Woo et al., 2015) and behavior selection (Baliki and Apkarian, 2015) in chronic pain states, while functional impairments due to chronic pain have been reported in the prefrontal cortex (Apkarian et al., 2004; Metz et al., 2009), striatum (Baliki et al., 2012), and hippocampus (Mutso et al., 2012; Vachon-Presseau et al., 2013).

We discovered that increased pain avoidance was associated with decreased phosphorylation of Synapsin^{S9} and CREB¹³³ in the dmPFC. Phosphorylation of Synapsin at serine 9 by PKA causes its dissociation from synaptic vesicles, which promotes presynaptic neurotransmitter release (Chi et al., 2001; Czernik et al., 1987; Hosaka et al., 1999). CREB is a transcription factor that regulates the expression of target genes following phosphorylation at serine 133 (Impey et al., 2004; Shaywitz and Greenberg, 1999). Our results suggest that increased pain avoidance is associated with decreased pre-synaptic vesicle release in the dmPFC and a subsequent reduction in post-synaptic activation of CREB. Based on our findings, decreased dmPFC activity may promote pain avoidance in relation to negative affective states experienced in opioid withdrawal. Indeed, our results are in close accordance with a recent optogenetic study that found reduced dmPFC activity following peripheral nerve injury in association with avoidance of a previously pain-paired environment (Zhang et al., 2015). In contrast, we found that greater pain avoidance-like behavior in individual animals was associated with increased phosphorylation of Synapsin^{S9} and autophosphorylation (Thr286) of CaMKII in the vmPFC, suggesting that greater pain avoidance is associated with increased pre-synaptic vesicle release and autonomous activation of CaMKII in the vmPFC.

The differential association between pain avoidance and phosphorylation events in dmPFC and vmPFC may further be explained by the dissociable effects of these brain areas on stress/affective regulation. Activation of the dmPFC inhibits stress responses, while activation of the vmPFC promotes stress signaling (George & Koob, 2010). Lesions to the dmPFC increase stress responses through activation of the hypothalamic-pituitary-adrenal (HPA) axis, which is responsible for initiating the physiological stress response in mammals (Radley et al., 2006). In contrast, lesions to the vmPFC decrease stress responses through activation of the HPA axis (Radley et al., 2006). It is likely that our observed intracellular signaling changes interact with specific stress-related neurotransmitter systems to mediate pain avoidance behavior. For example, using a conditioned place avoidance model of post-traumatic stress disorder, Schreiber and colleagues discovered a higher density of corticotropin-releasing factor (CRF) cells in the vmPFC to be positively correlated with avoidance-like behavior (Schreiber et al., 2017). In the same study, CRF1 receptor antagonism in the vmPFC reduced avoidance of the stress-paired context. In future studies,

we plan to investigate how CRF signaling in dmPFC vs. vmPFC contributes to alterations in pain sensitivity on the mechanical conflict-avoidance task. Such investigations may provide further insight into the possible use of CRF1 receptor antagonists for the treatment of both chronic pain opioid dependence (McGinn and Edwards, 2016), as CRF1 receptor antagonism has proven effective in concomitantly reducing both escalated opioid intake and hyperalgesia (Park et al., 2015).

Interestingly, we discovered that greater pain avoidance-like behavior is associated with increased phosphorylation of TH^{S40} in the VS, but decreased phosphorylation of TH^{S40} in the DS of individual animals. We also found that increased pain avoidance was associated with decreased phosphorylation of CaMKII^{Thr286} in the VS. Tyrosine hydroxylase is the rate-limiting enzyme involved in dopamine biosynthesis, activated via serine 40 phosphorylation in a negative feedback fashion when there is a deficit of dopamine (Molinoff & Axelrod, 1971). In addition, CaMKII regulates dopaminergic activity in the striatum by regulating dopamine biosynthesis and dopamine transport (Sutoo et al., 2002; Fog et al., 2006; Steinkellner et al., 2012; Kawaai et al., 2015; Muller et al., 2016). Accordingly, our findings indicate that regulation of striatal dopamine signaling is closely associated with pain avoidance-like behavior. Indeed, there is evidence that low dopaminergic activity is associated with high pain sensitivity in humans (Treister et al., 2009). In addition, decreases in dopaminergic activity in the brain are thought to mediate negative affective states. Inhibition of A10 dopamine neurons projecting to the VS elicits a negative affective-like state and generates a place aversion in rodents (Liu et al., 2008). The neurobiological mechanisms underlying negative reinforcement processes also include decreased function of brain reward systems (e.g., the dopamine depletion hypothesis; Koob et al., 2014). Striatal dopamine receptors, in particular, have a key role in mediating reinforcement in addition to their role in pain processing. For example, mesolimbic dopamine signaling is thought essential for the negative reinforcing effects of analgesia in pain states (Navratilova et al., 2012), while Cahill and colleagues have proposed restoration of mesolimbic dopamine signaling as a therapeutic strategy for treating the negative affective dimension of chronic pain (Taylor et al., 2016). Activation of striatal dopamine receptors can suppress pain responses in animals, an effect prevented by administration of a dopamine antagonist (Lin et al., 1981; Ansah et al., 2007).

Based on our findings, it is possible that lower levels of dopamine in the VS (as reflected by less feedback inhibition of pTH⁴⁰) are associated with greater pain avoidance-like behavior during the mechanical conflict-avoidance task. It has previously been reported that morphine increases extracellular dopamine concentrations in the VS (Di Chiara and Imperato, 1988), while withdrawal from morphine decreases VS dopamine levels (Rossetti et al., 1992). Furthermore, there is evidence that inflammatory pain suppresses the rewarding properties of morphine by decreasing dopamine in the VS (Narita et al., 2005). This reward tolerance could actually lead to an escalation of opioid use, as animals experiencing chronic inflammatory pain prefer to self-administer higher levels of opioids (Hipólito et al., 2015). Our results are consistent with the theory that alterations of reward brain systems (e.g., TH activation) may contribute to the emergence of negative affective pain states (Mitsi and Zachariou, 2016). In future studies, we plan to directly measure alterations in dopamine

levels and dopamine receptor signaling in the DS vs. VS during the mechanical conflict-avoidance task.

In the HIP, we found that pain avoidance-like behavior was associated with increased phosphorylation of ERK in the dorsal hippocampus of individual animals. ERK is a kinase in the mitogen-activated protein kinase (MAPK) family that regulates synaptic plasticity (Impey et al., 1999) and several addiction-related processes (Zamora-Martinez and Edwards, 2014). Activation of ERK has also been implicated in central processing of nociceptive information (Cao et al., 2009) and conditioned place avoidance (CPA) (Wang et al., 2012). CPA is a sensitive measure for assessing aversive motivational states associated with opioid withdrawal (Azar et al., 2003), and ERK activation is involved in the development and extinction of CPA. Repeated exposure to opioid withdrawal-induced CPA increases phosphorylation of ERK in the dorsal hippocampus (Wang et al., 2015), while ERK activation in the anterior cingulate cortex is required for formalin pain-induced CPA (Cao et al., 2009). These findings indicate that central brain ERK activity is necessary for the development and expression of negative affect-conditioned states. Based on our findings, it is also possible that the same cellular mechanisms that are initiated in the dorsal hippocampus during contextual memory formation may dictate behavioral responses to avoid noxious stimuli during the mechanical conflict-avoidance task.

CONCLUSIONS

In summary, the present study demonstrates that rats in acute withdrawal from chronic morphine administration exhibit increased pain avoidance-like behavior, a measure that exhibited significant concurrent validity with Von Frey tests of mechanical hypersensitivity. This study supports previous evidence relating the generation of negative affective states experienced during opioid withdrawal to the promotion of escalated opioid intake to alleviate pain in a negative reinforcement fashion. Our findings also describe differential relationships between pain avoidance-like behavior and region-specific phosphorylation of Synapsin, CREB, CaMKII, TH, and ERK. These data provide evidence that specific alterations in cortical and subcortical activity may promote pain avoidance in the context of negative affective states, and contributes to our understanding of opioid-induced pain avoidance and the cognitive/motivational dimension of pain. As such circuitry is heavily impacted by chronic or excessive opioid exposure, further interrogation of within- and between-circuit neuroadaptations is warranted to better understand the pathological intersection of pain and addiction (Shurman et al., 2010; Cahill et al., 2016). We propose the continued use of the mechanical conflict avoidance assay for the development of novel therapeutic strategies for pain (Borsook et al., 2014). Investigations that shed light on individual differences in opioid and pain sensitivity and mesolimbic signaling may also help us maximize the beneficial use of opioid analgesics while minimizing addiction liability.

Acknowledgments

This work was generously supported by research and training grants from the National Institute on Alcohol Abuse and Alcoholism (T32AA007577, ARP; R00AA020839, SE) and by LSUHSC School of Medicine start-up funds.

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Figure 1.

The mechanical conflict-avoidance task is a non-reflex-based method that was employed to model increases in the motivation to avoid pain during opioid withdrawal. The mechanical conflict-avoidance apparatus contains three red acrylic chambers: a brightly lit (aversive) start chamber, a probe chamber of adjustable probes heights (0 mm, 0.5 mm, 1 mm, 2 mm, 3 mm, 4 mm, 5 mm), and a dark (rewarding) goal chamber. Training consisted of rats being given a choice between remaining in a lighted box or crossing over elevated probes (noxious stimulus) to reach a goal box. In this model, a longer latency to exit onto the probes is thought to reflect an increased motivation to avoid pain.

Figure 2.

(A) Experimental timeline. After initial training, animals were given 7 days of mechanical conflict-avoidance testing to assess baseline measurement of avoidance and to split rats into two equivalent groups. Rats were given two weeks of either escalating morphine (week 1-10 mg/kg, week 2-20 mg/kg) or saline injections every 24 hours to induce opioid dependence. Tests of mechanical hypersensitivity were conducted at the end of week 1 and week 2. To assess mechanical conflict-avoidance in morphine-dependent rats compared to saline controls, the stimulus-response assessment was repeated after two weeks of daily injections. During testing, rats received an additional week of either morphine (20 mg/kg) or saline injections. Behavioral measurements for each day were taken 24 hours after the previous drug injection (acute withdrawal in morphine-dependent rats). All animals were euthanized 24 hours after the final drug injection and brains were immediately dissected and snap-frozen in preparation for regional tissue sample collection. (B-D) Schematic representation of sub-regional brain samples collected (Paxinos and Watson, 1998). (B) DM = dorsomedial prefrontal cortex; (B) VM = ventromedial prefrontal; (C) DS = dorsal striatum; (C) VS = ventral striatum; (D) HIP = hippocampus.

Figure 3.

Von Frey testing. Rats were given two weeks of either escalating morphine (week 1: 10 mg/kg, week 2: 20 mg/kg) or saline injections every 24 hours to induce opioid dependence. Tests of mechanical hypersensitivity (von Frey) were conducted at the end of week 1 and week 2. In morphine-dependent animals in acute withdrawal, there was a significant decrease in von Frey thresholds between weeks 1 and 2 in (A) experimental group 1 (* $p < 0.05$) and (B) experimental group 2 (** $p < 0.01$), indicating heightened mechanical hypersensitivity. In saline controls, there was no difference in the von Frey thresholds between weeks 1 and 2 in (C) experimental group 1 ($p > 0.05$) and (D) experimental group 2 ($p > 0.05$).

Figure 4.

Mechanical conflict-avoidance testing. Rats were given two weeks of injections of either an escalating dose regimen of morphine (to induce opioid dependence) or saline. Rats were then given a choice between remaining in a lighted chamber or crossing over elevated probes to reach a goal chamber. There was a longer latency to exit onto the probes in morphine-dependent rats compared to controls (* $p<0.05$, *** $p<0.0001$ main effect of group across all probe heights; ### $p<0.0001$ main effect of probe heights) in experimental group 1 (A), group 2 (B), and groups 1 & 2 combined (C). (D) Changes in mechanical sensitivity (Von Frey test) over the week prior to the mechanical conflict avoidance testing negatively correlated with individual levels of subsequent pain avoidance-like behavior ($r=-.4413$; $p<0.05$). Black dots represent morphine-dependent rats in 24-hour withdrawal, while grey dots represent saline controls.

Figure 5.

Neurobiological correlates of pain avoidance-like behavior in the dorsomedial prefrontal cortex (dmPFC). **(A)** Pain avoidance was negatively correlated with phosphorylation of Synapsin^{S9} in the dmPFC ($r = -0.7569$; $p < 0.05$). **(B)** Pain avoidance was negatively correlated with phosphorylation of CREB^{S133} in the dmPFC ($r = -0.6493$; $p < 0.05$). Black dots represent morphine-dependent rats in 24-hour withdrawal, while grey dots represent saline controls.

Figure 6.

Neurobiological correlates of pain avoidance-like behavior in the ventromedial prefrontal cortex (vmPFC). **(A)** Pain avoidance was positively correlated with phosphorylation of Synapsin^{S9} in the vmPFC ($r=0.7645$; $p<0.05$). **(B)** Pain avoidance was positively correlated with phosphorylation of CaMKII^{Thr286} in the vmPFC ($r=0.6835$; $p<0.05$). Black dots represent morphine-dependent rats in 24-hour withdrawal, while grey dots represent saline controls.

Figure 7.

Neurobiological correlates of pain avoidance-like behavior in the striatum. **(A)** Pain avoidance was positively correlated with phosphorylation of TH^{S40} in the ventral striatum ($r=0.7495$; $p<0.05$). **(B)** Pain avoidance was negatively correlated with phosphorylation of CaMKII^{Thr286} in the ventral striatum ($r=-0.7881$; $p<0.05$). **(C)** Pain avoidance was negatively correlated with phosphorylation of TH^{S40} in the dorsal striatum ($r=-0.6774$; $p<0.05$). Black dots represent morphine-dependent rats in 24-hour withdrawal, while grey dots represent saline controls.

Figure 8.

Neurobiological correlates of pain avoidance-like behavior in the hippocampus. Pain avoidance was positively correlated with phosphorylation of ERK in the dorsal hippocampus ($r=0.7223$; $p<0.05$). Black dots represent morphine-dependent rats in 24-hour withdrawal, while grey dots represent saline controls.

Neurobiological Correlates of Pain Avoidance-Like Behavior in Reward- and Pain-Related Brain Regions

Figure 9.

Summary of observed neurobiological correlates of pain avoidance-like behavior. Our findings suggest that increased pain avoidance is associated with decreased pre-synaptic transmission capacity (pSynapsin) and a subsequent reduction in post-synaptic activation of CREB in the dmPFC. In contrast, increased pain avoidance is associated with increased pre-synaptic vesicle release (pSynapsin) and a subsequent increase in post-synaptic activation of CaMKII in the vmPFC. Weakened dmPFC activity disinhibits downstream stress responses, while strengthened vmPFC activity facilitates the stress response. Accordingly, increases in PFC-mediated stress signaling may facilitate pain sensitivity on the mechanical conflict-avoidance task. Our findings suggest that increased pain avoidance is also associated with lower levels of dopamine in the VS (as reflected by less feedback inhibition on pTH⁴⁰ and decreased activation of CaMKII), but higher levels of dopamine in the DS (as reflected by greater feedback inhibition of pTH⁴⁰), indicating that regulation of striatal dopamine signaling is closely associated with pain avoidance. Finally, increased pain avoidance is associated with increased phosphorylation of ERK in the dorsal hippocampus. Since ERK activity is necessary for the development and expression of negative affect-conditioned states, activation of ERK may dictate appropriate contextual responses to avoid noxious stimuli during the mechanical conflict-avoidance task.

Correlation matrix of region-specific protein phosphorylation and pain avoidance-like behavior in morphine-dependent and non-dependent animals (r-values)

Table 1

Brain Region	pERK	pCaMKII	pCREB	pSynapsin	pTH
Dorsomedial PFC	-0.4948	-0.5783	*-0.6493	*-0.7569	-0.1143
Ventromedial PFC	-0.2613	*0.6835	0.2174	*0.7645	-0.2246
Ventral Striatum	-0.0157	*-0.7881	0.0926	-0.4976	*0.7495
Dorsal Striatum	0.2575	-0.3492	-0.3938	0.2006	*-0.6774
Hippocampus	*0.7223	0.3341	0.5344	-0.4485	-0.6121

* Significance: p<0.05