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Short communication

Hippocampal and amygdalar increased BDNF expression in the extinction of opioid-induced place preference

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ABSTRACT

The opioid crisis was exacerbated during the COVID-19 pandemic in the United States with alarming statistics about overdose-related deaths. Current treatment options, such as medication assisted treatments, have been unable to prevent relapse in many patients, whereas cue-based exposure therapy have had mixed results in human trials. To improve patient outcomes, it is imperative to develop animal models of addiction to understand molecular mechanisms and identify potential therapeutic targets. We previously found increased brain derived neurotrophic factor (bdnf) transcript in the ventral striatum/nucleus accumbens (VS/NAc) of rats that extinguished morphine-induced place preference. Here, we expand our study to determine whether BDNF protein expression was modulated in mesolimbic brain regions of the reward system in animals exposed to extinction training. Drug conditioning and extinction sessions were followed by Western blots for BDNF in the hippocampus (HPC), amygdala (AMY) and VS/NAc. Rears, as a measure of withdrawal-induced anxiety were also measured to determine their impact on extinction. Results showed that animals who received extinction training and successfully extinguished morphine CPP significantly increased BDNF in the HPC when compared to animals deprived of extinction training (sham-extinction). This increase was not significant in animals who failed to extinguish (extinction-resistant). In AMY, all extinction-trained animals showed increased BDNF, regardless of behavior phenotype. No BDNF modulation was observed in the VS/NAc. Finally, extinction-trained animals showed no difference in rears regardless of extinction outcome, suggesting that anxiety elicited by drug withdrawal did not significantly impact extinction of morphine CPP. Our results suggest that BDNF expression in brain regions of the mesolimbic reward system could play a key role in extinction of opioid-induced maladaptive behaviors and represents a potential therapeutic target for future combined pharmacological and extinction-based therapies.

Introduction

Opioid addiction is one of the leading causes of death in the United States (Hedegaard et al., 2020). Since the Covid-19 pandemic began in 2020, a spike of more than 25,000 annual overdose related deaths from 2018 to 2020 was reported. Over 60% of these overdoses were opioid related, now accounting for over 1% of all deaths in the United States (Ahmad et al., 2021). Currently, opioid addiction and its withdrawal symptoms are treated with a combination of behavioral therapies and medication assisted treatment (MAT). While MAT modalities have been conventionally used to prevent opioid relapse and overdose, the use of behavioral approaches are needed to ensure long-term abstinence (Maglione et al., 2018; SAMHSA, 2021). For example, in cue-based exposure therapy, the subjects are exposed to previous drug-associated contexts/cues to weaken the link between these cues and the drug, thereby extinguishing the drug-associated memories and preventing relapse (Mellentin et al., 2017; Everitt, 2014; Torregrossa and Taylor, 2013). However, the current extinction-based therapies have shown limited clinical success, perhaps due to lack of a deeper understanding of the molecular and biological mechanisms underlying extinction (Millan

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et al., 2011). For this purpose, we have previously reported that the extinction of morphine-induced conditioned place preference (CPP) in rats, through receiving extinction training, displayed a different expression profile for synaptic plasticity genes of the ventral striatum/nucleus accumbens (VS/NAc), as compared with rats receiving withdrawal (i.e., forced abstinence in their home-cages). Particularly, our results showed a significant increase in brain derived neurotrophic factor (bdnf) mRNA in rats who successfully extinguished morphine CPP (Martínez-Rivera et al., 2019), suggesting this neurotrophin as a potential molecular mediator for extinction of drug-associated conditioned stimuli. BDNF is a neurotrophic growth factor that has been linked to extinction processes in fear (Rosas-Vidal et al., 2018) and drugs of abuse (Bobadilla et al., 2019; Castino et al., 2018; Règue-Guyon et al., 2018). Multiple studies in opioid addiction have shown extensive associations between BDNF and extinction of morphine (Koo et al., 2012). In fact, rats that were chronically exposed to heroin, decrease BDNF expression (Li et al., 2017). These effects seem to involve BDNF's activity through its receptor Trk-B, as rats infused with its antagonist (ANA-12) impairs extinction of morphine CPP (Jorjani et al., 2021). Similarly, inhibition of accumbal BDNF signaling impairs extinction of cocaine seeking, while exogenous BDNF microinjections facilitate this process (Bobadilla et al., 2019). Also, BDNF has been associated with extinction of nicotine and alcohol-related behaviors (Barker et al., 2015).

Gene expression can be a transient process that do not necessarily translate to long-term differences in protein expression within the targeted area and its circuits (Vélez-Bermúdez and Schmidt, 2014). Thus, while we previously showed an increase of bdnf mRNA in the VS/NAc, it remains to be determined if the bdnf transcript is translated to its mature protein, and whether the abundance in the VS/NAc and other interconnected regions of the mesolimbic reward circuit are modulated. Given that VS/NAc has low BDNF expression (Conner et al., 1997; Li et al., 2013), it is possible that this neurotrophin can be transported through VS/NAc afferents (Altar et al., 1997) expressing high levels of BDNF such as the hippocampus (HPC) and amygdala (AMY) (Conner et al., 1997). Therefore, in this study we combined morphine-CPP followed by extinction training to interrogate BDNF protein expression in the mesolimbic reward system. We further compared the level of BDNF expression in rats displaying different extinction phenotypes (i.e., success or failure). To test for anxiety-related behaviors associated with drug withdrawal we measured the frequency of rears according to extinction phenotype. Understanding the molecular milieu of the extinction of drug-related behaviors in preclinical models of addiction represents a step closer to develop combined pharmacological and behavioral treatments that facilitate abstinence in patients suffering from opioid addiction.

Materials and methods

Subjects and drug

Adult male Sprague Dawley rats (~350 g; Envigo Laboratories., Indianapolis, IN) were individually housed with food and water available *ad libit*um (12:12 h light/dark cycle; 64°F, 30% humidity). Behavioral experiments were performed during the light phase of the cycle, and procedures were in accordance with the IACUC of the University of Puerto Rico, Medical Sciences Campus. Morphine sulfate (Sigma Aldrich, St. Louis MO; 5 mg/kg) was dissolved in saline (0.09%; 0.2 ml/100 g of body weight) and administered subcutaneously to all experimental groups. This dose of 5 mg/kg was chosen as previous studies found that it is sufficient to induce morphine-place preference without impacting mobility (Martínez-Rivera et al., 2019; Heinrichs et al., 2010; Mueller et al., 2002), and able to elicit withdrawal-induced anxiety (Zhang and Schulteis, 2008), as demonstrated by them when a repetitive 5 mg/kg dose was administered by 4 days, resembling early stages of drug-dependence.

Conditioned place preference (CPP)

Protocols for morphine conditioning and extinction were performed as previously described (Martínez-Rivera et al., 2019); Fig. 1 A, adapted from Martínez-Rivera et al., 2019). Animals were habituated in the behavioral chamber for 20 min (Day 1). A 20-min test was performed to determine side preference (baseline; Day 2). They were then morphine-conditioned for 45 min over an 8-day period (Days 3–10) with alternating morphine and saline injections. Next day (Day 11; conditioning test), animals were allowed to move freely between compartments for 1 h, and their side preference was determined as measured by the percentage of time spent in the drug-paired side. Increased percentage of time as compared to baseline was considered as the conditioning index. Forced extinction (Heinrichs et al., 2010; Leite-Morris et al., 2014), in which animals were restricted to the drug-associated side for 1 h in the absence of drug, was performed on Days 12–15. Next day (Day 16; extinction test), animals were allowed to move freely between compartments for 20 min, and their side preference was determined. Decreased percentage of time in the drug-paired side as compared to the conditioning test was considered as an index of extinction. Animals that exhibited high CPP (below 20% reduction in preference for the drug paired side) after extinction training were categorized as extinction resistant. In sham-extinction, animals did not receive extinction training and were kept in their home cages (Fig. 1A). Behavioral data was acquired using the Any-Maze tracking system (Stoelting Co., Wood Dale, IL).

Rears as withdrawal-induced anxiety

Drug-conditioned rats are likely to experience withdrawal. In animal studies, anxiety-related behaviors are used to assess drug-withdrawal as they are easily observable (Sarnyai et al., 1995; Lu et al., 2005). Rearing, defined as an animal standing on its hind legs has been commonly used as a measure of anxiety in the absence of fear (McNaughton and Gray, 2000; Myers and Carlezon, 2010), although it is highly influenced by novelty (Lever et al., 2006). In this study, we measured the frequency of rears as indicative of withdrawal-induced anxiety in animals that received either extinction training (extinction and extinction-resistant groups) or sham-extinction. Scores of the behaviors were performed from video recordings during a period of 20-min and compared at three time points: baseline, conditioning test day, and extinction test day. Hand scores were observer-blind regarding animal group or treatment.

Western blots

Western blots and tissue dissection were performed as previously described (Martínez-Rivera et al., 2015; Sharma and Fulton, 2013). Reagents were purchased to Bio-Rad Laboratories, CA. In brief, animals were sacrificed within 1 h of behavioral testing (Day 16), and the VS/NAc, HPC and AMY were dissected, homogenized and total protein concentration determined. Samples were denatured and loaded in a 4–20% SDS polyacrylamide gel. After electrophoresed, proteins were transferred into nitrocellulose membranes and verified by Ponceau's staining. Blotto blocking buffer (5% milk protein) was applied to the membranes and incubated overnight with primary anti-BDNF rabbit monoclonal antibody (# ab108319, Abcam, MA) using the following dilutions: HPC, 1:2000; AMY and VS/NAc; 1:1000). Secondary mouse anti-rabbit IgG-HRP antibody was used at 1: 2000 (Santa Cruz Biotechnology, TX). Primary anti-ß-actin rabbit polyclonal antibody (# ab8227; Abcam, MA) was used for normalization (1:5000). Blots were visualized using an enhanced chemiluminescence kit (SuperSignal Femto, Pierce IL) and images obtained using a VersaDoc 1000 system (Bio-Rad, CA). Western blots were performed in triplicate technical replicates from three (AMY) or four (HPC, VS/NAc) independent experiments. Densitometric analysis was performed using NIH ImageJ software (v1.47d).

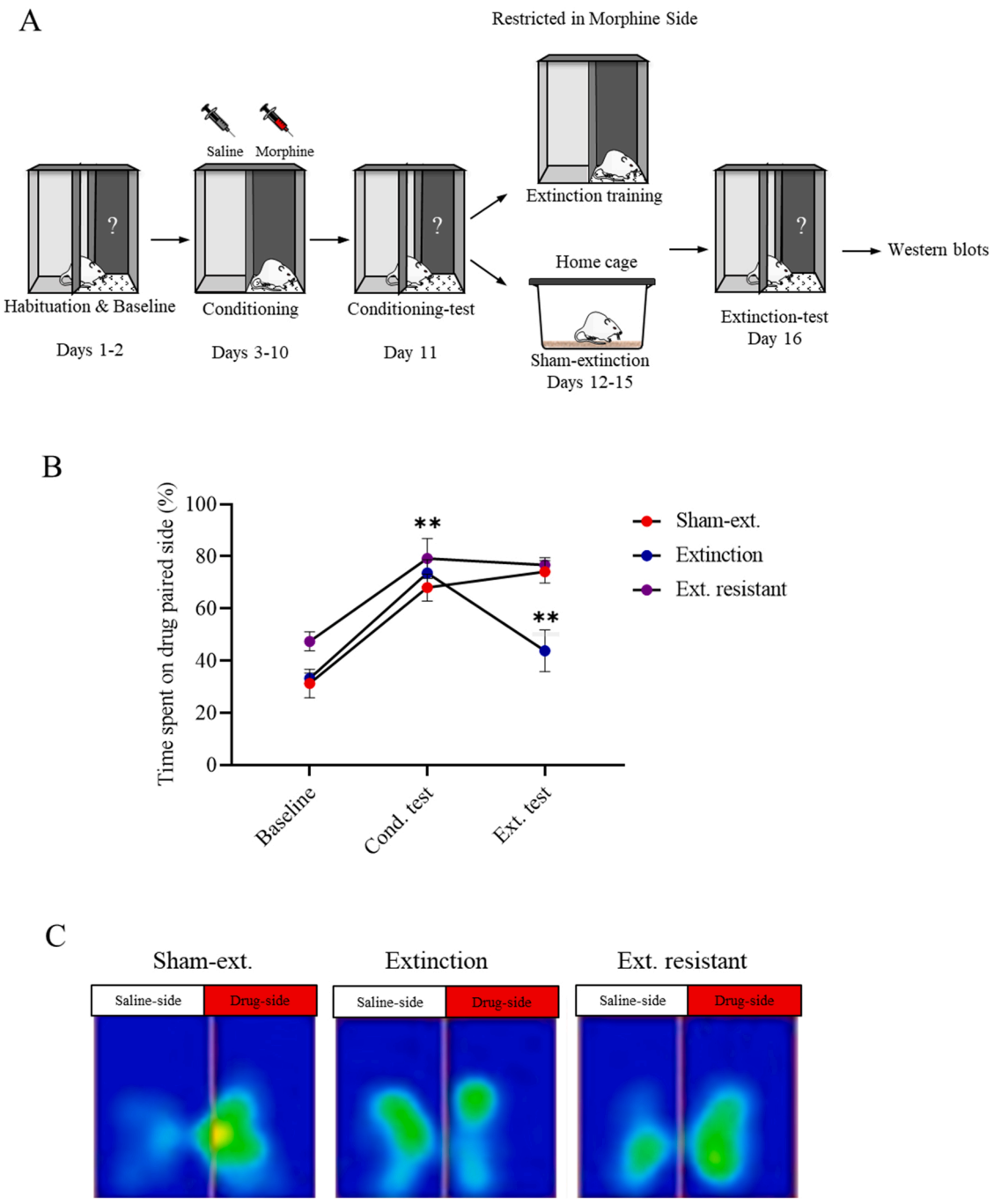

Fig. 1. Extinction of morphine CPP. **(A)** Diagram showing the protocol for morphine conditioning and extinction (adapted from Martinez-Rivera et al., 2019). **(B)** A pre-conditioning test (baseline) established the non-preferred (drug-paired) side for each animal. Conditioning and extinction tests were performed after conditioning and extinction training, respectively. Two subgroups within the extinction-trained animals were observed: animals that extinguished morphine CPP (Extinction) and animals that resisted extinction (Ext. resistant). Animals in the sham-extinction (Sham-ext.) group did not receive extinction training. **(C)** Representative heatmaps showing time spent on either drug or saline-paired side during extinction-test day for animals in the Sham-ext., Extinction and Ext. resistant animal groups. ** $p < 0.01$. Sham-ext.: $n = 4$; Extinction: $n = 4$; Ext. resistant: $n = 4$. Data is shown as mean and SEM.

Statistical analysis

Data is presented as mean $\pm$ SEM and statistical significance established at $p < 0.05$. CPP behaviors were analyzed using Two-way repeated measure ANOVA, followed by Tukey post-hoc tests. Rears and Western blots were analyzed using one-way ANOVA, followed by Tukey post-hoc tests (GraphPad Prism 9.0).

Results

Conditioning and extinction of morphine place preference

To model extinction learning, rats were first conditioned to express drug place preference by morphine administration in an alternate pattern (morphine/saline), followed by 4 days of either extinction or sham-extinction sessions (Fig. 1A). Learning index after conditioning sessions was measured by increased time spent on the morphine-paired

side in the test day as compared to baseline. Conversely, extinction learning was determined by a reduction of time in the drug-paired side comparable to conditioning-test levels. Two-way repeated measures ANOVA showed significant effects in time (CPP Phases: baseline, conditioning test, extinction test) and groups (sham-extinction, extinction, and extinction-resistant); main effect of time: $F\ (1.5,\ 14) = 37$; $p < 0.0001$; main effect of group: $F\ (2,\ 9) = 9.5$; $p < 0.01$; interaction: $F\ (4,\ 18) = 3.6$; $p < 0.05$. Like our previous findings (Martínez-Rivera et al., 2019), results showed increased preference (CPP) for the morphine-paired side in all groups (Fig. 1B; Tukey post hoc; p's < 0.01). For extinction learning, we showed that animals in the extinction group significantly reduced their preference for the morphine-paired side (below 50% of time) when compared to the conditioning test (Fig. 1B; Tukey post hoc; p's < 0.01). This result contrast with animals that retained their preference for morphine in the extinction-resistant and sham-extinction groups (both behaved similarly [$p = 1.0$]), suggesting different rates of extinction learning. Indeed, heatmaps of representative animals in each experimental group showed that animals extinguishing morphine-place preference spent more time in the saline-paired side (Fig. 1C; green signal), as compared to animals in the sham-extinction and extinction-resistant groups. No significant differences were observed between individuals in each group at baseline or conditioning test (Fig. 1B; Tukey post hoc; p's > 0.05). Overall, we were able to capture individual phenotypes to facilitate the identification of molecular substrates signaling specific extinction-related behaviors.

Rears as withdrawal-induced anxiety

Withdrawal induced anxiety has the potential to interfere with the effectiveness of the extinction process (Lu et al., 2005; Myers and Carlezon, 2010). To determine whether withdrawal-related behaviors influenced the behavioral outcomes of extinction trained animals, we measured the frequency of rears which has been associated with opiate withdrawal (Azorlosa and Simmons, 1999; McKendrick et al., 2020). A one-way ANOVA showed a significant interaction between groups: $F\ (2,\ 41) = 26$, $p < 0.0001$) corresponding to a significant reduction in rears during conditioning (55 ± 2.7) and extinction tests (55 ± 3.8) as compared to baseline (79 ± 2.2) (Fig. 2A, Tukey post hoc: sham-extinction vs. extinction, $p < 0.0001$; sham-extinction vs. extinction-resistant $p < 0.0001$). To detect potential differences in extinction outcome between extinction-trained animals and those in their home cage (sham-extinction), we performed a separate analysis according to groups for the extinction test day. A one-way ANOVA showed a significant interaction between groups: $F\ (2,\ 9) = 8.5$, $p = 0.008$) which corresponds to a significant increase in the frequency of rears of the sham-extinction group (72 ± 2.3) compared to both extinction (52 ± 3.0) and extinction-resistant (46 ± 6.0) (Fig. 2B, Tukey post hoc: sham-extinction vs. extinction, $p = 0.029$; sham-extinction vs. extinction-resistant, $p = 0.007$). This increase in rears is similar to values seen at baseline ($p = 0.9769$) suggesting an environmental novelty effect. As the frequency of rears did not differ in animals in the extinction or extinction-resistant groups, our data suggest that withdrawal-induced anxiety is not a key factor for the extinction of opiate-related behaviors in early stages of drug dependence.

Changes in expression of BDNF

To determine whether extinction training led to changes in BDNF expression, we performed Western blots on tissue lysates from HPC, AMY, and VS/NAc. In the HPC, One-way ANOVA showed a significant effect of group: $F\ (2,\ 9) = 5.250$ $p = 0.0308$), and a significant interaction: $F\ (2,\ 9) = 5.250$; $p < 0.05$). The extinction group increased BDNF expression (230%) as compared to a normalized sham-extinction group (Fig. 3 A, Tukey post hoc; $p < 0.05$). A non-significant increase in BDNF was observed in the extinction-resistant group (173%; Tukey post hoc; $p = 0.2154$), although no significant difference was observed between

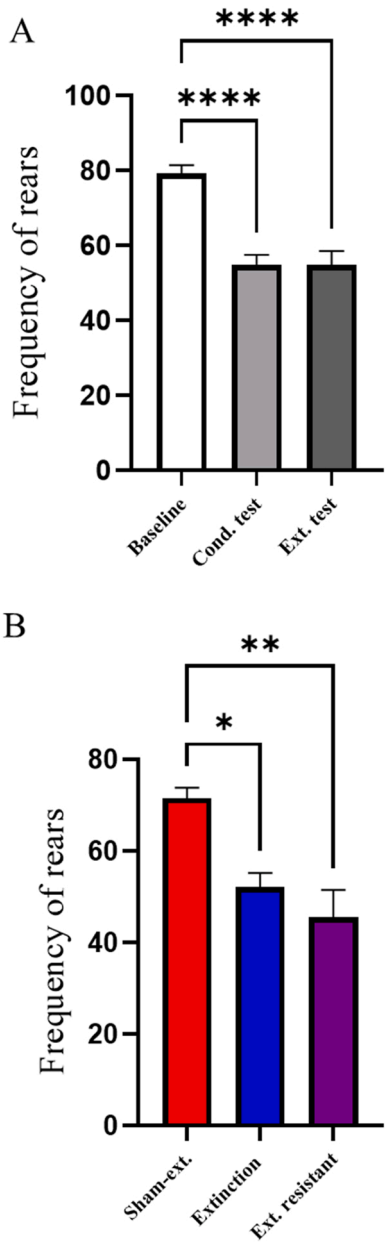

Fig. 2. Withdrawal-induced anxiety as measured by rears. **(A)** Frequency of rears at baseline, conditioning test (Cond. test) and, extinction test (Ext. test) timepoints. **(B)** Frequency of rears during extinction test in animals that received extinction training or sham-extinction. * $p < 0.05$, ** $p < 0.005$, **** $p < 0.0001$. Sham-ext.: n = 3; Extinction: n = 7; Ext. resistant: n = 6. Data is shown as mean and SEM.

extinction and extinction-resistant groups (Tukey post hoc; $p = 0.3791$). In AMY, One-way ANOVA showed a significant effect in group: $F\ (2,\ 6) = 8.99$ $p = 0.0156$) and a significant interaction: $F\ (2,\ 6) = 8.996$; $p < 0.05$). An increase in BDNF in both extinction (157%) and extinction-resistant (138%) groups was observed as compared to sham-extinction (Fig. 3B, Tukey post hoc; sham-extinction vs. extinction $p = 0.0191$; sham-extinction vs. extinction-resistant $p = 0.0323$). Analysis of the VS/NAc did not reveal any significant differences (Fig. 3 C). Increased expression of BDNF in HYP and AMY were specific to the mature protein, as Pro-BDNF showed no significant changes. These findings suggest that hippocampal BDNF may be an important molecular mediator for the extinction of conditioned opioid-related behaviors, whereas increased amygdalar BDNF may be encoding environmental cues when exposed to extinction training.

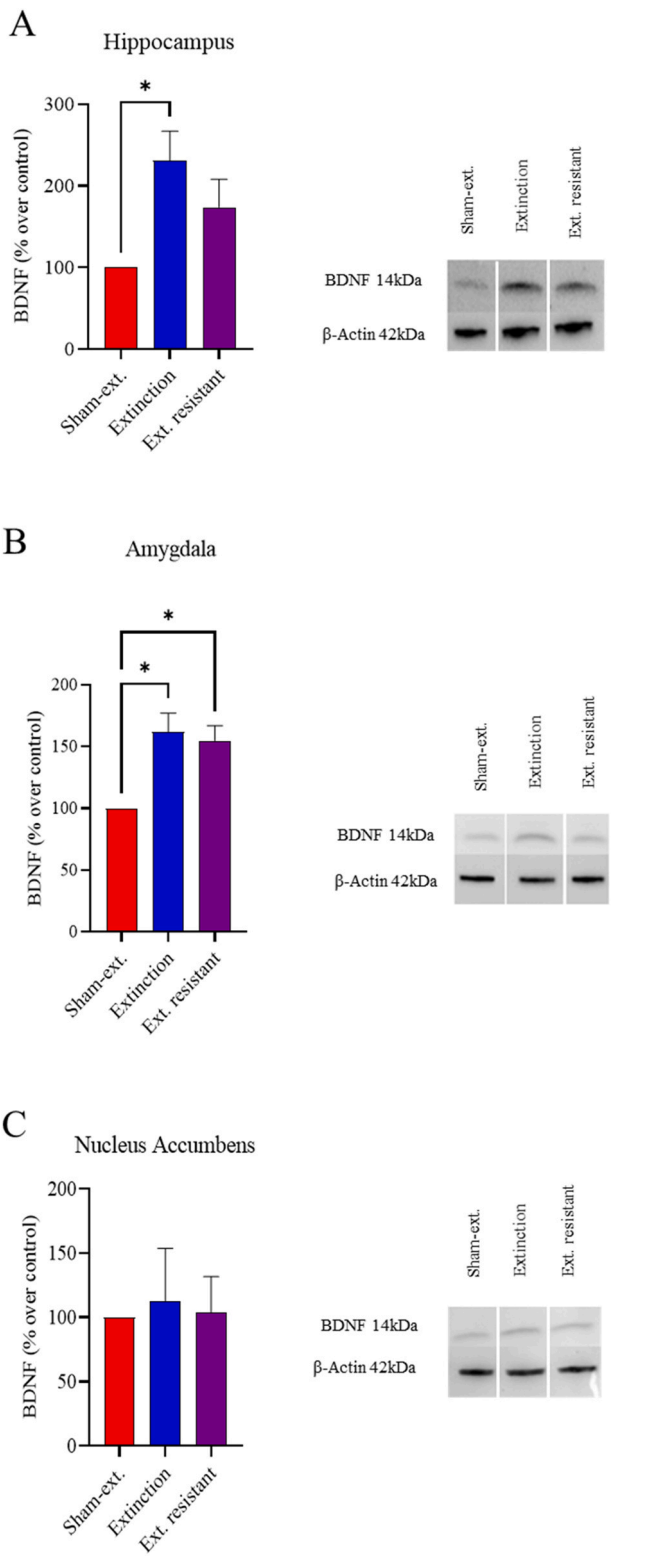

Fig. 3. The effect of morphine-extinction training in BDNF expression in the hippocampus, amygdala and VS/NAc. Densitometric analysis (left panel) and representative Western blots (right panel) of BDNF expression (% over control) in the hippocampus **(A),** amygdala **(B)** and nucleus accumbens **(C)** of animals that received extinction training (Extinction and Ext. resistant groups) or sham-extinction (Sham-ext.). * $p < 0.05$. Biological samples: Hippocampus and NAc n = 4; Amygdala n = 3. Technical replicates: three () for each group (Sham-ext., Extinction and Ext. resistant). BDNF (14 kDa-mature band). ß-actin was used for normalization. Data is shown as mean and SEM.

Discussion

Improving clinical outcomes in exposure-based therapies requires further study into the molecular mechanisms of extinction learning. Several brain regions, including the VS/NAc, HPC, and AMY, among others, are involved in the extinction of behaviors related to addiction (Goode and Maren, 2019). Our study provides continuity to previous molecular findings that implicates BDNF as a key player for extinction learning (Andero and Ressler, 2012; Cowansage et al., 2010; Martínez-Rivera et al., 2019). In the present study we assessed relative BDNF expression in these brain regions, in animals that extinguished morphine CPP, as compared to animals receiving sham-extinction, or those that were extinction-resistant. The most significant finding was a BDNF increase in HPC of rats that received extinction training and subsequently extinguished morphine CPP. This increase was the highest among all the studied regions and its value doubled the sham-extinction group.

BDNF in extinction circuits

In the VS/NAc, we found no significant differences in BDNF expression after extinction training for morphine in either the extinction or resistant groups. This result was surprising as the NAc has been extensively associated with extinction (Gibson et al., 2019; Dutta et al., 2021; Fatahi et al., 2020a). In fact, animals who extinguished morphine CPP significantly increased bdnf transcript in this region (Martínez-Rivera et al., 2019). Evidence of BDNF as a pro-extinction molecule in the VS/Nac comes from pharmacological studies in which the Trk-B antagonist, ANA-12, increased withdrawal symptoms (Rezamohammadi et al., 2020) and facilitated acquisition and expression of morphine CPP (Jorjani et al., 2021). Similarly, Trk-B knockdown in medium spiny neurons enhanced morphine CPP (Koo et al., 2014). Therefore, it is possible that the major changes of BDNF protein expression during extinction training are occurring in other sources which potentially target the VS/NAc. Inputs coming from prefrontal and hippocampal regions have been shown to transport BDNF to the VS/NAc (Conner et al., 1997; Giannotti et al., 2018). Moreover, the pro-extinction effect appears to be subregion specific (Chiara, 2002). For example, BDNF infusions to the NAc core decrease cocaine seeking (Bobadilla et al., 2019), while infusions to the NAc shell only increased reinstatement (Graham et al., 2007). As we examined the entire NAc region in our study, we do not rule out the possibility of missing subregion specific BDNF modulation, in particular the shell, which is more commonly associated with extinction (Gibson et al., 2019; Dutta et al., 2021). Post-translational modifications or afferent BDNF activity in the HPC and AMY are also plausible mechanisms that warrant further research.

Studies in fear learning were at the forefront in our understanding of extinction processes (Milad and Quirk, 2012) as they demonstrated that fear and addiction shared common extinction pathways (Myers et al., 2011; Peters et al., 2009). Fear studies also provided evidence for the importance of BDNF in extinction. In the HPC, reduced BDNF expression was observed in rats that failed extinction (Peters et al., 2010), while in the ventral hippocampus (vHPC), increased BDNF immunoreactivity was shown in rats that underwent extinction training (Rosas-Vidal et al., 2018). When infused in the vHPC of fear conditioned rats, BDNF also increased the firing rate of structures signaling extinction learning (i.e., infralimbic cortex) (Rosas-Vidal et al., 2014). The dorsal HPC also may be involved in addiction processes given its dense connections to the VS/NAc and its associations with behavioral sensitization (Degoulet et al., 2008). In addiction studies, extinction of cocaine self-administration increased expression of the BDNF receptor, Trk-B (Hastings et al., 2020), and morphine-trained rats increased hippocampal BDNF expression when naloxone-induced withdrawal was applied in a delay-based decision-making task (Fatahi et al., 2020b). In our study, both extinction and extinction-resistant animals showed increases in BDNF, although only the extinction group attained

significance. This increase is similar to previous findings from Martinez-Rivera et., al (2019), Rosas-Vidal et al. (2018) were Bdnf mRNA transcripts were only significantly increased in animals in the extinction group but not in the extinction-resistant. Nonetheless, it is possible that extinction-resistant animals may require additional training sessions to attain extinction. Whether BDNF is transported from the HPC to the VS/NAc, or to other brain regions, remains to be determined.

In AMY, our study showed that both extinction and extinction-resistant animals significantly increased BDNF expression. Like the HPC, the role of BDNF in AMY are scarce in addiction. However, studies in fear showed that AMY plays a critical role in context-based learning were connections from the vHPC to the basal amygdala contribute to encoding conditioned fear (Kim and Cho, 2020). In addition, AMY also regulates fear extinction mediated by signaling from the central nucleus and basolateral amygdala (Pare and Duvarci, 2012). It is suggested that regulation of fear extinction relies on BDNF signaling as TrK-B knock-down blocks consolidation of extinction (Chhatwal et al., 2006), Trk-B agonists facilitate extinction (Andero et al., 2011), and cortical BDNF/Trk-B signaling to the AMY is required for fear extinction learning (Meis et al., 2020). The role of amygdalar BDNF in extinction of addictive behaviors is understudied, though it may be regulating aversive responses to withdrawal (Heldt et al., 2014). Yet, opposing effects were reported for alcohol and opioids; having decreased BDNF in AMY during alcohol withdrawal (You et al., 2014), but an increase during morphine-induced withdrawal (Martínez-Laorden et al., 2020). However, decreased BDNF expression in alcohol withdrawal might be due to the absence of an extinction phase. As the AMY is associated with context-based learning, the increased amygdalar BDNF expression observed in our study is likely due to the extinction training, as it was present in both extinction and extinction-resistant groups. Given that these two groups did not differ in BDNF expression in the AMY after extinction training, it seems that successful extinction may be dictated by the HPC through a BDNFergic mechanism.

Behavioral symptoms associated to morphine-withdrawal

To determine whether differences in extinction outcomes were due to the severity of withdrawal-induced anxiety, we assessed rears at various time points of the protocol. Increased rearing activity has been previously associated with morphine conditioning (Lu et al., 2005), as well as other factors such as exposure to novel environments (Lever et al., 2006). In our study, we observed increased rears in baseline as compared to conditioning or extinction-tests, which may be due to an environmental novelty effect, as animals receiving extinction training had been exposed to the CPP chamber for 8 additional sessions. Similarly, during the extinction-test day, we observed and increase in rears in sham-extinction animals. This could also be a novelty effect, as this group showed no increase in rears during the conditioning test day (no drug was administered) suggesting the increase was not due to lack of the expected morphine. Previous studies have shown that reintroducing animals to the context restores the effects of novelty (Lever et al., 2006). In our study, extinction trained animals showed no differences in rears despite extinction phenotype, suggesting that differences in extinction between groups are not based on severity of drug-withdrawal, at least during early stages of drug dependence.

Conclusion

A growing number of studies suggest that interfering with maladaptive emotional memories may lead to better treatment outcomes for diseases (Liu et al., 2020). As BDNF has been suggested as a potential treatment for addiction (Barker et al., 2015), our findings show that extinction training elicits changes in BDNF expression in the HPC and AMY, although only the HPC seems to dictate successful extinction learning. Differences in successful extinction vs extinction-resistant are not likely due to withdrawal-induced anxiety. Future studies will interrogate downstream effector molecules of the BDNF signal transduction cascade, such as Trk-B and pERK. Therefore, our preclinical model of addiction suggests that BDNF could play a key role in the extinction of opioid-related behaviors that combined with behavioral therapies could be a potential pharmacological target for addiction.

CRediT authorship contribution statement

Mario E. Lloret-Torres: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Jennifer L. Barreto-Estrada:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing. **Roxsana N. Ayala-Pagan:** Data curation, Investigation, Visualization, Project administration. **Freddyson J. Martinez-Rivera:** Conceptualization, Investigation, Validation, Writing – review & editing. **Bonilla Pedro:** Data curation, Formal analysis, Investigation, Writing – original draft.

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