Chapter

Expression and Extinction of Morphine-Related Aversive Memories in Mice: Underlying Neural Substrates and Impact of Morphine Encapsulation in Liposomes

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Abstract

The conditioned-place aversion (CPA) paradigm is a widely used animal model for determining the dysphoric and aversive properties of withdrawal, as well as to study the neural substrates underlying the aversive memory associated with drug withdrawal. Therefore, one of the aims of the present chapter was to investigate possible strain differences in morphine-withdrawn mice after CPA training. Different studies have shown that corticotrophin-releasing factor (CRF) is critically involved in aversion-driven associative learning. So, we also measured the effects of CP-154,526, a selective CRF1 receptor (CRF1R) antagonist, on CPA activation and extinction and on brain-derived neurotrophic factor (BDNF) expression in dentate gyrus (DG) and basolateral amygdala (BLA), two limbic areas that play a critical role in emotional learning and memory. In the last section of the chapter, we show our main results concerning the effects of encapsulating morphine in liposomes on CPA expression and extinction. Our results indicate a crucial role for CRF, through CRF1R, in molecular changes involved in aversive memory expression and extinction demonstrating that CRF1R antagonists might be a potential therapeutic target in drug addiction. Finally, morphine encapsulation in liposomes could mitigate the progress of addiction and constitute a good option for the therapeutic use of this drug.

Keywords: conditioned-place aversion (CPA), strain, morphine encapsulation, brain-derived neurotrophic factor (BDNF), hypothalamic–pituitary-adrenocortical (HPA) axis

1. Introduction

Morphine is an opiate thoroughly used for acute and chronic pain control, as oncologic pain [1]. Besides, opioids are increasingly being prescribed for chronic non-oncologic pain and for mild to moderate outpatient pain, even though the data on their efficacy and safety for this indication advise against it [2].

It is well known that chronic opioid administration can trigger physical dependence and addiction. Nowadays, opioid use disorder is a major public health problem, related to an important morbidity and mortality [3]. In this sense, prescription opioid misuse and abuse have led to a serious opioid crisis in the last years [4].

Although previous research has shown that the addictive process is a heritable psychiatric disease [5–7], the genetic substrates of opioid addiction are not yet well known. For that, different authors have suggested that genetic components are crucial for changes in opioid liking/disliking, causing the special progress of opioid addiction in rodents [8].

Substances of abuse activate the hypothalamic–pituitary-adrenocortical (HPA) axis after withdrawal, through the stimulation of corticotropin-releasing factor (CRF) in the paraventricular nucleus (PVN) of the hypothalamus, which results in an intense discharge of adrenocorticotropic hormone (ACTH) and corticosterone [9]. Corticosterone mediates somatic and negative affective-like factors of withdrawal [10–13] and CRF is involved in reward- and aversion-related associative learning [14]. Nevertheless, it is not completely elucidated if it is due to common or separated mechanisms that can be dissociated.

The faster a drug enters the central nervous system, the higher its addictive potential is, maybe because of the more euphoric and/or reinforcing properties. As mentioned before, opioids are drugs with a great capacity to induce addiction. New drug delivery systems, such as liposomes and nanoparticles, are being developed to beat the limitations of the traditional drug delivery formulations. These systems can improve the molecular properties of already available drugs, minimize drug degradation, prevent harmful side-effects and induce a controlled drug release [15, 16]. Although these transporters have been widely studied for years in the field of pain release [17, 18], little is known about the effects of encapsulating morphine on addiction [19, 20].

It has been demonstrated that the rewarding attributes of substances of abuse are in part related to their ability to strengthen memory consolidation. New treatments for the addictive process include the chance of managing learning and memory to diminish drug seeking by inducing abstinence and limiting relapse [21]. The conditioned-place aversion (CPA) paradigm is a widely used animal model for determining the dysphoric and aversive properties of withdrawal [22]. Besides, this test has been considered as a measure for drug-seeking behavior and allows research concerning the neural substrates related to the negative memory associated with drug withdrawal [23]. Briefly, in CPA test, opioid withdrawal is cue-linked to the environment [22] so, the aversive signs that appear during withdrawal syndrome are associated with the place where animals undergo the negative experience. If conditioning has occurred, when animals are returned to the paired context in a drug-free state, they will avoid it.

Extinction of this aversion occurs when animals are repeatedly exposed to the withdrawal-related environment without the unconditioned stimulus and finishes when animals no longer avoid the cue-associated context. The aversive memory associated with drug withdrawal can recall motivational and/or emotional feelings

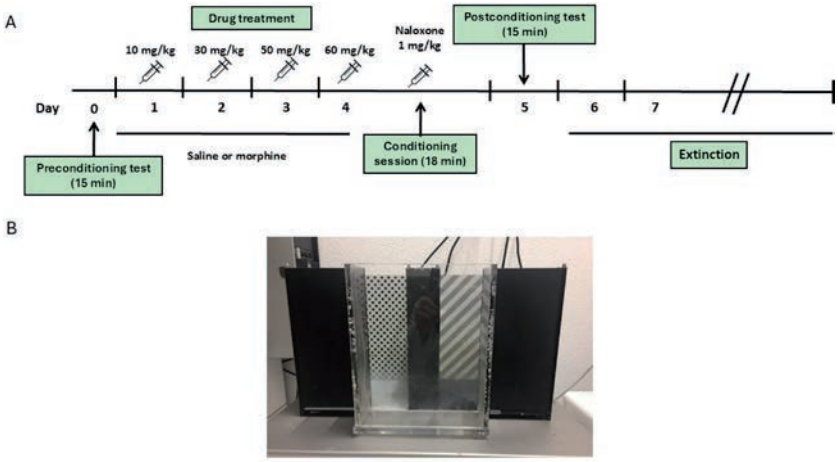

Figure 1.
Schematic representation of the conditioned-place aversion (CPA) protocol (A). Photograph of the CPA equipment (B).

that manage compulsive drug taking [24, 25]. Thus, extinction of this memory needs associative learning, consolidation and formation of a new memory [26–28] and this process has been proposed as a therapeutic strategy for drug addiction treatment.

Figure 1 shows the equipment and the general experimental protocol used by our group for the CPA paradigm, which is divided into five phases: preconditioning test, drug treatment, conditioning session, postconditioning test and extinction.

2. Differences between Swiss and B6 mice in CPA expression and extinction: Role of CRF1 receptor in these processes

There is considerable evidence indicating the involvement of HPA axis in addiction-like behaviors. However, little is known about strain differences in morphine-withdrawn animals after a CPA training. To approach this issue, our laboratory designed a novel study, including two mice strains usually manipulated in research: Swiss and C57BL/6 J (B6), which show different locomotor responses to opioids [29]. B6 mouse strain is a widely used strain of inbred mice to determine behavior and its response to different nociceptive stimuli is considered within the normal range [30]. Therefore, we selected this strain to be compared with Swiss as a prototype of outbred stock. Strain surveys of common inbred and outbred mice are an effective manner to obtain information about the genetic involvement in intricate disorders [31].

Research has suggested substrain differences in CPA with a motionless object like a mouse (a toy mouse) [32] or in naloxone-induced CPA [8]. However, the mechanisms underlying these differences are nowadays not completely understood. Thus, it is important to understand the genetic and neurobehavioral basis of opioid aversion because it could help physicians in the prevention and treatment of opioid dependence.

Body weight loss induced by naloxone-administered morphine withdrawal is an unbiased and precise measure of opioid withdrawal [33, 34] and our group has studied this sign in Swiss and B6 mice (**Figure 2**). Three-way analysis of variance (ANOVA) showed significant main effects on body weight loss for acute

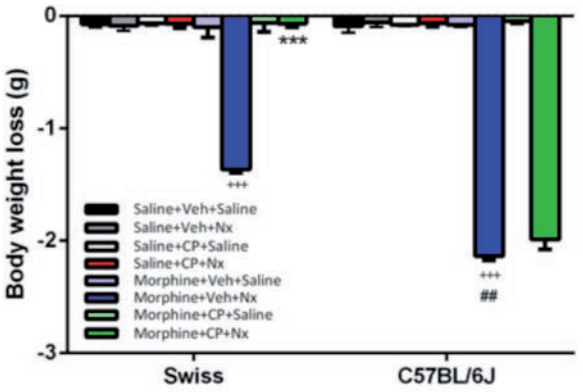

Figure 2.
*Effect of naloxone (Nx, 1 mg/kg, s.c.) on body weight loss in Swiss and C57BL/6 mice administered with morphine (10–60 mg/kg, i. p.) or saline every 12 h for 4 days. CP-154,526 (CP) or its vehicle (Veh) was injected 30 min before naloxone. Data are the mean ± SEM. N = 16 for each group, +++p < 0.001 versus Saline+Veh + Nx; ***p < 0.001 versus Morphine+Veh + Nx; ##p < 0.05 versus Swiss mice. Adapted from [35].*

treatment, chronic treatment, and for strain. We also observed main effects for the following interactions: chronic treatment and acute treatment; strain and acute treatment; strain and chronic treatment; and, finally, for the interaction of the three factors (strain, chronic treatment and acute treatment). Naloxone administration to morphine-treated animals induced a significant increase in body weight loss in Swiss and B6 mice, being the weight loss in morphine-withdrawn B6 mice significantly higher than the one observed in Swiss mice after withdrawal. These results suggest a more severe withdrawal in B6 mice. The body weight loss percentages were 3.3% for Swiss and 7.9% for B6 mice. CP-154,526, a selective CRF1 receptor (CRF1R) antagonist, administration blocked the increased body weight loss in Swiss mice, with no changes in B6 mice. These results point to the involvement of CRF1R in the body weight loss observed in Swiss mice. However, CRF1R does not seem to have a role in B6 mice [35].

Our research group has also studied the effects of strain, morphine and naloxone on place aversion induced by morphine withdrawal (**Figure 3**). Three-way ANOVA revealed significant main effects for acute treatment, chronic treatment, and for strain. Main effects for the following interactions were also shown: chronic treatment and acute treatment; strain and acute treatment; strain and chronic treatment and, finally, for the interaction of the three studied factors (strain, chronic treatment and acute treatment).

We were also interested in studying if CPA acquisition was accompanied by increased corticosterone plasma levels. Thus, we measured this glucocorticoid levels in Swiss and B6 mice after naloxone-induced CPA expression and CPA extinction (**Figure 4**). Two-way ANOVA for corticosterone plasma levels after CPA expression revealed a main effect of strain, treatment and strain x treatment interaction, including a significant positive correlation between plasma corticosterone levels and the place aversion score ($r^2 = 0.3832$, $p = 0.0002$, **Figure 4C**). With respect to corticosterone after CPA expression, two-way ANOVA revealed significant effect of strain, treatment and strain x treatment interaction. As shown in **Figure 4A**, naloxone increased ($p < 0.001$) corticosterone plasma levels after CPA expression in morphine-administered

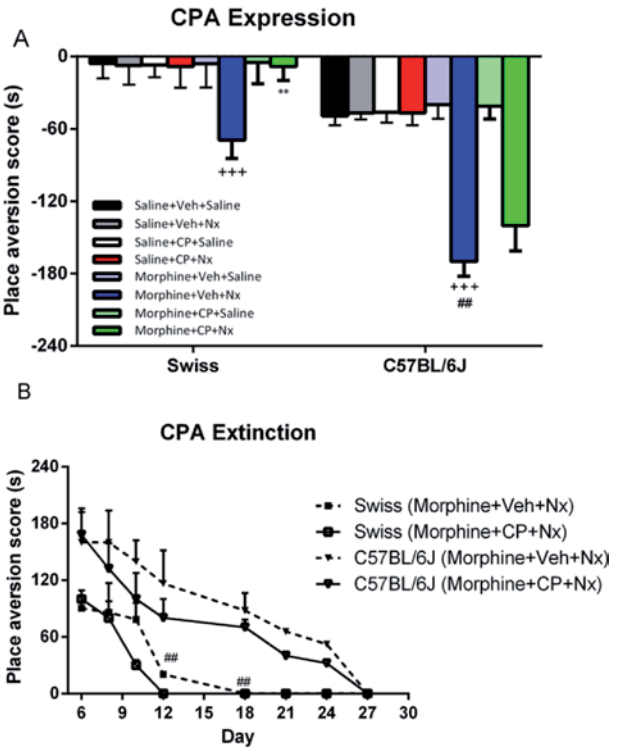

Figure 3.
*Conditioned-place aversion (CPA) expression induced by naloxone (Nx, 1 mg/kg, s.c.) in Swiss and C57BL/6 mice injected with morphine or saline. CP-154,526 (CP) or its vehicle (Veh) was administered 30 min before naloxone. The score was calculated for each mouse as the difference between the postconditioning and the preconditioning time spent in the drug-paired compartment (A). Extinction of CPA training. Aversion scores from day 5 to 27 are represented (B). Data are expressed as the mean ± SEM. N = 8 for each group, +++p < 0.001 versus saline+veh + nx; **p < 0.01 versus Morphine+Veh + Nx; ## p < 0.01 versus Swiss mice. Adapted from [35].*

Swiss mice versus the saline+vehicle+naloxone group or B6 mice treated with morphine+vehicle+naloxone. Nevertheless, no changes in corticosterone plasma levels were observed after CPA expression in B6 mice. CP-154,526 significantly antagonized the increased expression of corticosterone observed in Swiss mice after CPA expression. On the other hand (**Figure 4B**), extinct morphine-withdrawn Swiss mice showed a decrease in corticosterone plasma levels versus the same strain treated with saline and B6 mice treated with morphine. The selective CRF1R antagonist significantly decreased the corticosterone plasma levels versus the group treated with morphine+vehicle+naloxone. No changes were observed in corticosterone levels in B6 mice after naloxone-conditioned withdrawal or after CP-154,526 administration.

Comparisons between Swiss and B6 mice showed that different neurobiological mechanisms might be influencing the retrieval and extinction of aversive memories. Such differences in the behavioral response could be partly based on intrinsic differences in the ability to associate (learning) and recall (memory) aversive events. Significant strain differences in different learning tasks (Y-maze, object recognition and passive avoidance) have been shown in C57 and BALB animals [36]. Thus, genetic variability could influence addictive behaviors, although these cognitive differences remain to be explained.

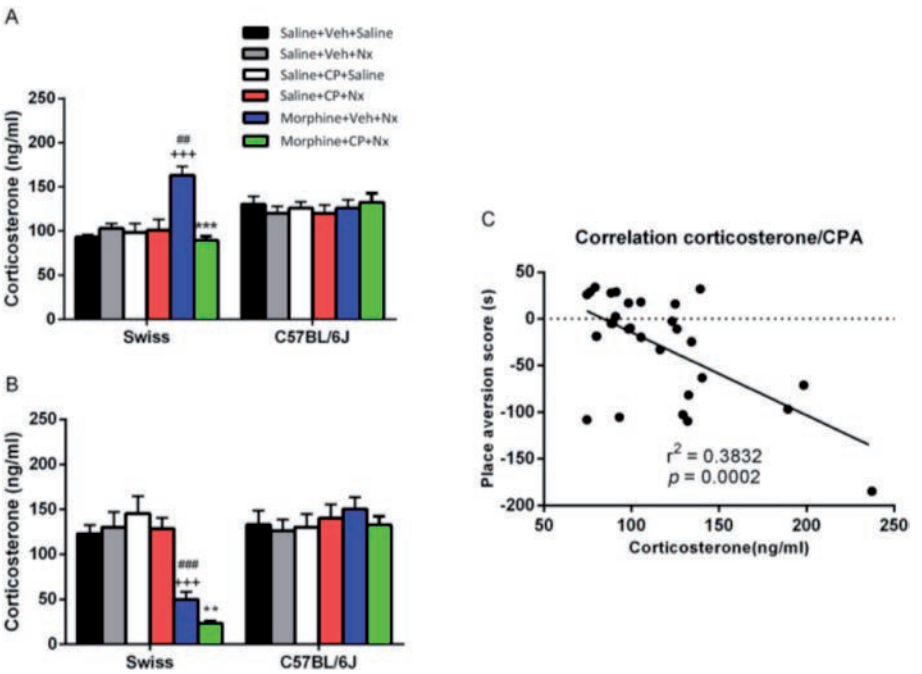

Figure 4.
*Corticosterone plasma levels after expression (A) and extinction (B) of conditioned place aversion (CPA) training. (C) Correlation between corticosterone and CPA. Data are expressed as the mean ± SEM. N = 8 for each group, +++p < 0.001 versus Saline+Veh + Nx; ++p < 0.01, ***p < 0.001 versus Morphine+Veh + Nx, ##p < 0.01, ###p < 0.001 versus B6 mice. CP: CP-154,526. Nx: Naloxone. Adapted from [35].*

3. Involvement of BDNF signaling pathway in the aversive effects of morphine: Role of CRF1 receptor

Brain-derived neurotrophic factor (BDNF) is one of the most distributed and extensively studied neurotrophins in the mammalian brain. It regulates neuron synaptic plasticity and plays a crucial role in learning and memory [37]. Besides, growing evidence shows that BDNF can regulate glucocorticoid receptor signaling and HPA axis function in depression through a crosstalk mechanism [38]. However, the possible link between HPA axis and BDNF in the creation and extinction of aversive memories associated with conditioned morphine withdrawal remains not well understood. For this reason, our group was interested in studying the possible changes in proBDNF and BDNF after CPA expression and extinction in two limbic areas that play a critical role in emotional learning and memory, dentate gyrus (DG) and basolateral amygdala (BLA) [39, 40]. In both DG (**Figure 5**) and BLA (**Figure 6**), the Western blotting (WB) analysis revealed that BDNF expression was increased after CPA expression compared to their controls, with no changes in proBDNF expression [41].

These results are in agreement with studies from other laboratories describing that the BDNF signaling pathway in the rat amygdala is required for the formation of aversive memories associated with conditioned morphine withdrawal [42]. Besides, CRF1R antagonism by the administration of CP-154,526 was able to block the observed increase in BDNF expression, with no effect on proBDNF expression. The fact that CP-154,526 treatment reduced BDNF expression to basal levels in parallel

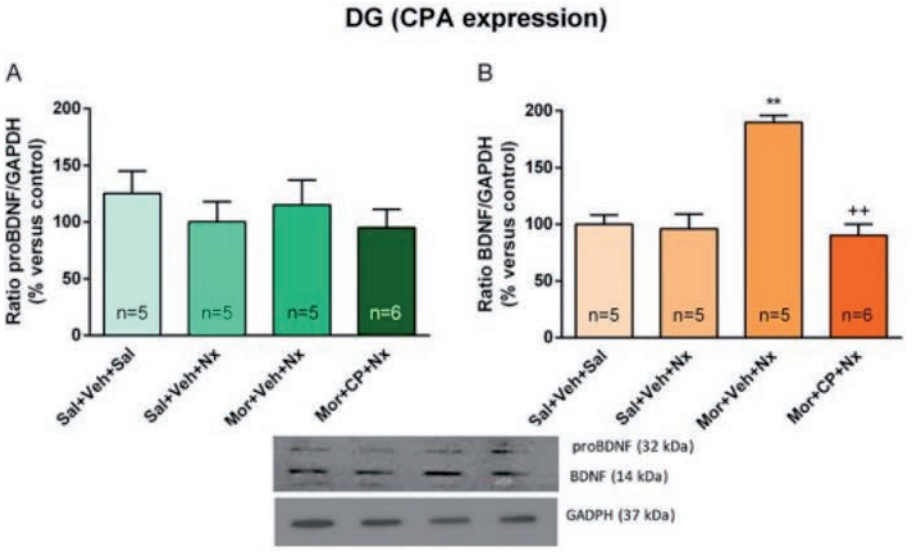

Figure 5.
*Western blotting analysis of pro-brain-derived neurotrophic factor (proBDNF) (A) and BDNF (B) in dentate gyrus (DG) after conditioned place aversion (CPA) expression. Each bar represents the mean optical density ± standard error of the mean (SEM). Values are expressed as % of control. **p < 0.01 versus saline (Sal) + vehicle (veh) + naloxone (Nx); ++p < 0.01 versus morphine (Mor) + veh + Nx. Adapted from [41].*

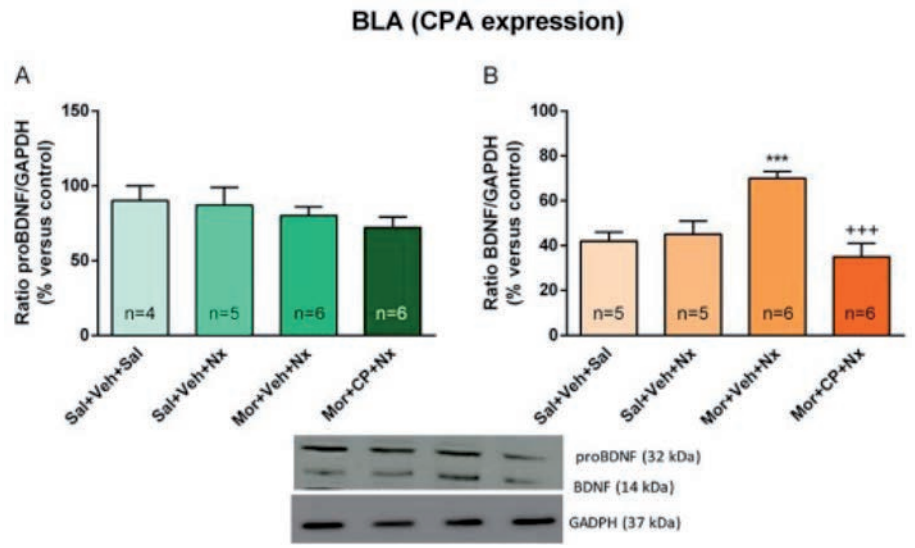

Figure 6.
*Western blotting analysis of pro-brain-derived neurotrophic factor (proBDNF) (A) and BDNF (B) in basolateral amygdala (BLA) after conditioned place aversion (CPA) expression. Each bar represents the mean optical density ± standard error of the mean (SEM). Values are expressed as % of control. ***p < 0.001 versus saline (Sal) + vehicle (veh) + naloxone (Nx); +++p < 0.001 versus morphine (Mor) + veh + Nx. Adapted from [41].*

with plasma corticosterone levels (shown in **Figure 4**) may indicate that BDNF is linked to HPA axis activity in the formation of aversive memory.

Concerning proBDNF and BDNF expression after CPA extinction, our laboratory has shown that BDNF levels were significantly decreased in DG (**Figure 7**) after CPA

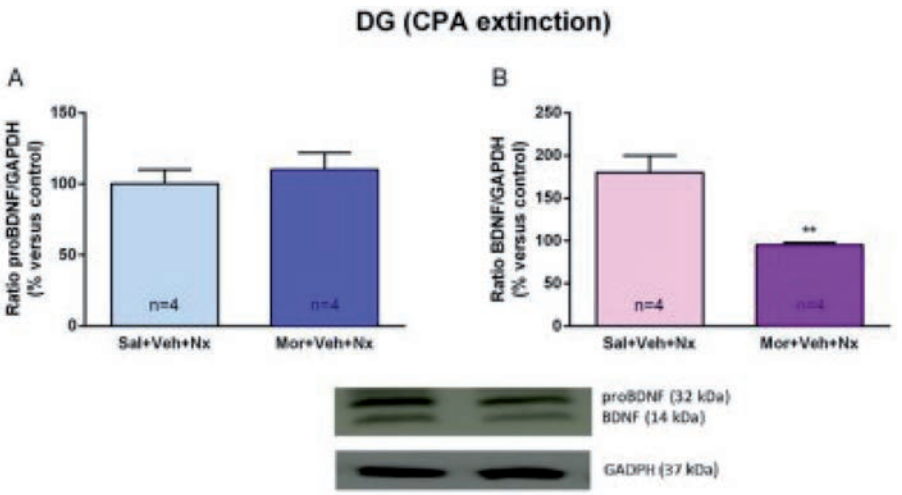

Figure 7.
*Western blotting analysis of pro-brain-derived neurotrophic factor (proBDNF) (A) and BDNF (B) in dentate gyrus (DG) after conditioned place aversion (CPA) extinction. Each bar represents the mean optical density ± standard error of the mean (SEM); values are expressed as % of control. **p < 0.01 versus saline (Sal) + vehicle (veh) + naloxone (Nx). Adapted from [41].*

extinction in morphine-withdrawn animals when compared to the chronic saline-treated group injected with naloxone, with no changes in proBDNF. Similar results were obtained in BLA (**Figure 8**).

ProBDNF has demonstrated an effect contrasting with that of BDNF in processes such as substance use disorder [43], so studies often use the BDNF/proBDNF or proBDNF/BDNF ratio to express their results. This ratio could provide important information about the prevalence of negative or positive neuromodulatory mechanisms.

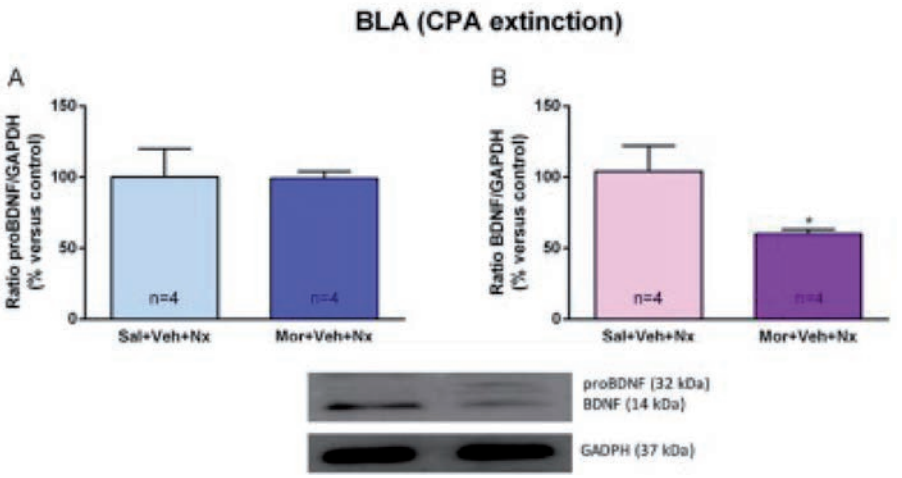

Figure 8.
*Western blotting analysis of pro-brain-derived neurotrophic factor (proBDNF) (A) and BDNF (B) in basolateral amygdala (BLA) after conditioned place aversion (CPA) extinction. Each bar represents the mean optical density ± standard error of the mean (SEM). Values are expressed as % of control. *p < 0.05 versus saline (Sal) + vehicle (veh) + naloxone (Nx). Adapted from [41].*

Altogether, our results suggest that the increase in HPA axis activity following conditioned morphine withdrawal alters BDNF levels in areas involved in learning and memory. Aversive memory was extinguished after CPA expression when corticosterone levels and BDNF expression in DG and BLA were declined to basal levels. We also show that blockade of CRF1 signaling abolished CPA expression and the increase in corticosterone release and BDNF expression observed after conditioned morphine withdrawal, suggesting a link between HPA axis and BDNF through CRF1R.

4. Liposome-encapsulated morphine facilitates extinction of aversive memories

Liposomes are spherical vesicles composed of one or more concentric lipid bilayers that enclose an aqueous core for drug encapsulation [44]. Liposomes and other nanotransporters, such as nanoparticles, allow the development of drug formulations with reduced drug toxicity, prolonged drug effectiveness, and, sometimes, more specific delivery [45]. Liposomes have been used in clinical practice for several years and have been demonstrated to be effective and safe for human use (Doxil ®, AmBisome ®). Not long ago, a morphine sulfate extended-release liposome-based drug (DepoDur®) was approved by the Food and Drug Administration (FDA). Opioid formulations capable of being administered systemically with longer release characteristics might be useful in chronic pain treatment. Therefore, it is worth to examine techniques to improve stability and release profile of liposomal opioid formulations [46].

Although liposomes have been deeply examined for many years for pain therapy [17], little is known about the effects of encapsulating morphine on addiction [20]. Thus, our group was interested in producing different drug release formulations for morphine encapsulation to study the cellular and molecular neuroadaptive changes that occur in the brain reward and in the brain stress system during morphine dependence and withdrawal, using these carriers. Among these controlled-release formulations, we developed multillamelar liposomes (MLV) and pegylated unilamellar liposomes (PEG-L) to encapsulate morphine. The pharmacokinetics study showed that morphine encapsulation, either in PEG-L or MLV, resulted in a controlled release of the entrapped drug [47].

First, we measured the physical signs of morphine withdrawal in MLV- or PEG-L-injected animals and in animals administered with morphine in its free form. For this purpose, on day 4 of the CPA schedule, animals were weighed before and 18 min after naloxone injection. Their behavior was videotaped for 18 min. The experimenter analyzed the recording to assess behavioral patterns. A score was calculated using a modification of the scale described by Gellert and Holtzman (Gellert and Holtzman, 1978). Graded signs were: weight loss (each 1% of weight loss evaluated as 1); number of jumps (1–4 evaluated as 1, 5–9 evaluated as 2, and > 10 evaluated as 3); and number of body shakes (1–2 evaluated as 2, >3 evaluated as 4). Checked signs (only presence or absence was evaluated) were diarrhea and ptosis. Interestingly, our results show that PEG-L-treated animals manifested fewer signs of physical dependence (**Figure 9**).

Second, we demonstrated that encapsulating morphine in liposomes decreases CPA duration but not CPA expression. As can be seen in **Figure 10**, morphine administration, both in its free form or included in MLV or PEG-L, induced a significant place aversion to the naloxone-paired chamber compared to the saline group

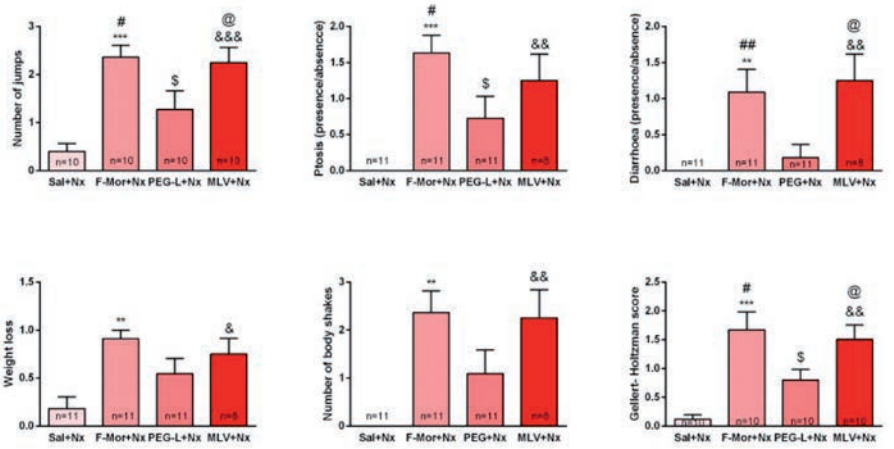

Figure 9.
*Signs of morphine withdrawal and Gellert-Holtzman score in animals treated with morphine for 15 min after the conditioning sessions with naloxone. Data are the mean ± SEM. **p < 0.01, ***p < 0.001, $p < 0.05, &p < 0.05, &&p < 0.01, &&&p < 0.001 versus S + Nx; #p < 0.05, ##p < 0.01, @p < 0.05 versus PEG-L + Nx. Saline (S), naloxone (Nx), free morphine (F-M). Adapted from [47].*

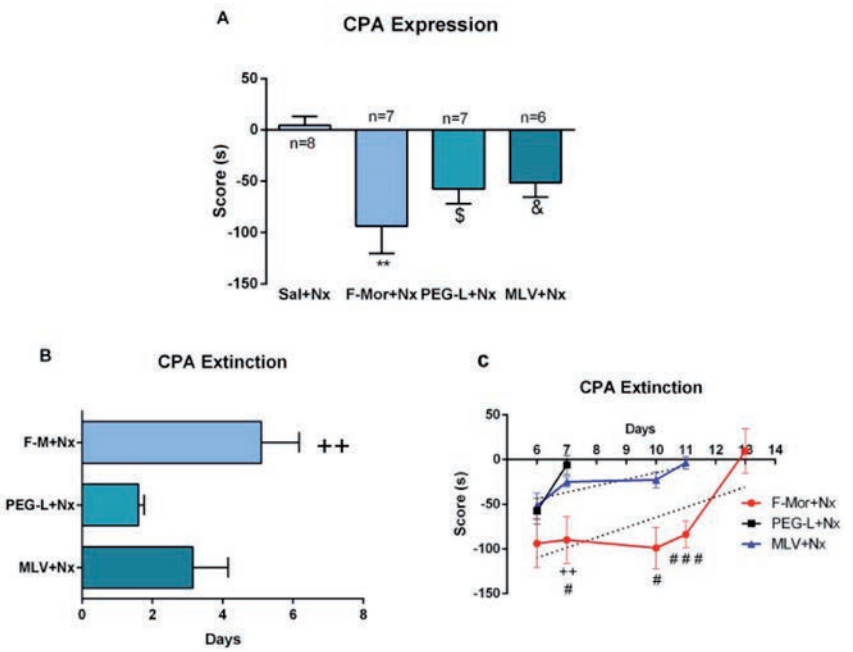

Figure 10.
*Effect of saline or morphine administration on conditioned place aversion (CPA) paradigm. Mice were injected with chronic morphine/saline for 4 days and received an acute injection of naloxone (1 mg/kg, s.c.). Morphine was administered encapsulated into MLV or PEG-L or in its free form (10–60 mg/kg, n = 8 per group) The score was calculated for each mouse as the difference between postconditioning and preconditioning time spent in the naloxone-paired compartment (A). Maintenance of the CPA induced by a s.c. injection of naloxone to mice treated chronically with morphine, MLV, or PEG. The bars represent the number of days that each animal needed to achieve complete extinction of CPA (defined as a lack of significant differences with respect to preconditioning values). Data are the mean ± SEM. ++p < 0.01 versus PEG-L + Nx (B). Extinction of CPA training. Aversion scores from day 6 to 13 are represented. Data are the mean ± SEM. **p < 0.01, $p < 0.05, &p < 0.05 versus Sal+Nx; ++p < 0.01 versus PEG-L + Nx; #p < 0.05, ###p < 0.01 versus MLV + Nx. Saline (S), naloxone (Nx), free morphine (F-Mor) (C). Adapted from [47].*

(**Figure 10A**). Moreover, one-way ANOVA for the CPA score showed no differences between free morphine, MLV, and PEG-L. Mice administered with free morphine presented aversion for the naloxone-paired compartment for a longer time (5.10 days) than MLV (3.14 days) and PEG-L (1.60 days) (**Figure 10B**). One-way ANOVA showed a significant increase in extinction days in the free-morphine group compared to PEG-L, and with no differences between MLV and PEG-L (**Figure 10B**). These results also indicate that the time necessary to extinguish CPA was 1.5- fold higher in the free-morphine group, compared to MLV, and threefold higher than PEG-L. Two-way ANOVA showed a main effect of kind of formulation and day, with a significant interaction between kind of formulation and day. Besides, *post hoc* analysis displayed a significant increase in the aversion maintained on day 11 in free morphine-administered animals compared to MLV-treated mice (**Figure 10C**).

5. Conclusions

CPA expression and extinction causes neuroadaptive changes in the HPA axis in a strain-specific way. These processes yield alterations in the activity of HPA axis through CRF1R in Swiss mice, which could provoke the differences in aversion and in the shorter extinction period observed in this stock. Therefore, the genetic variability in the memory processing of drug aversion could be a result of a specific individual vulnerability to drug addiction. Besides, BDNF and corticosterone are basic for the construction of aversive memory and a possible link between HPA axis and BDNF is suggested through CRF1R. Overall, these results indicate a crucial role for CRF, through CRF1R, in molecular changes involved in aversive memory expression and extinction demonstrating that CRF1R antagonists might be a potential therapeutic target in drug addiction.

On the other hand, results concerning morphine encapsulation in liposomes point to a significant advantage of using this nanotransporter, since this technological procedure would make morphine a less addictive drug, as shown in memory consolidation results. This novel and original line of research needs much more work in order to build stronger conclusions but, based on our preliminary results, we might conclude that the encapsulation process could mitigate the progress of addiction and constitute a good option to the therapeutic use of drugs like morphine.

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Conflict of interest

The authors declare no conflict of interest.

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