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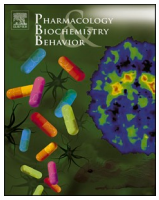

Context evoked morphine conditioned effects can be equivalent to morphine induced drug effects in terms of behavioral response and ERK activation in reward associated subcortical brain structures

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ABSTRACT

Conditioned drug cues can evoke brief drug-like responses. In this report we show that using brief test sessions, contextual cues can induce conditioned hyperlocomotion and ERK responses equivalent to morphine induced responses. To assess acute unconditioned effects, rats that received morphine (MOR-1) or vehicle (VEH-1) were immediately placed onto an arena for a 5-min locomotion recording session after which ERK was measured in the ventral tegmental area (VTA) and nucleus accumbens (NAc). There were no differences in locomotion between the groups. However, the MOR-1 group had strong ERK activation in VTA and NAc. To assess MOR-conditioned effects, a chronic phase was carried out according to a Pavlovian conditioning protocol. There were two MOR paired groups (MOR—P), one MOR unpaired (MOR-UP) group and two VEH groups. The treatments were administered over 5 daily five minute test sessions. The final conditioning test was on day 6, in which one of the MOR-P groups and one of the VEH groups received VEH (MOR-P/VEH-6 and VEH/VEH-6, respectively). The other MOR-P group and VEH group received MOR (MOR-P/MOR; VEH/MOR-6, respectively). The MOR-UP group received VEH (MOR-UP/VEH-6). Rats received the treatments immediately prior to a 5-minute arena test, and after the session ERK was measured. No morphine induced locomotor stimulation was observed on day 1 but on days 2 to 5, hyperlocomotion in both MOR-P groups occurred. On test day 6, the MOR-P/VEH-6 and the MOR-P/MOR-6 groups had comparable locomotor stimulant responses and similar ERK activity in the VTA and NAc. The MOR-UP group did not differ from the VEH group. We suggest that ERK activation evoked by acute morphine served as a Pavlovian unconditioned stimulus to enable the contextual cues to acquire morphine conditioned stimulus properties and increase the incentive value of the contextual cues.

1. Introduction

It has been extensively demonstrated in rodents that repeated morphine treatments produce potent stimulant effects manifested in spontaneous motor activity behavior as hyperactivity (Vezina and Stewart, 1984; Neisewander and Bardo, 1987; Vanderschuren et al., 1999; Powell and Holtzman, 2001; Tzschentke and Schmidt, 1995; Leite Júnior et al., 2019). These morphine effects are consistent with a large number of studies in rodents that have shown that drugs which have pro-dopamine effects such as amphetamine (Robinson, 1984; Mazurski and Beninger, 1987; Anagnostaras et al., 2002; Singer et al., 2014), cocaine (Carey and Gui, 1998; Horger et al., 1999; Zavala et al., 2000; Carey and Damianopoulos, 2006; Todtenkopf and Carlezon, 2006) and apomorphine (Mattingly and Gotsick, 1989; Damianopoulos and Carey, 1993; Võikar et al., 1999; Keller et al., 2002; Bloise et al., 2007; de Matos et al., 2010; Santos et al., 2017) induce locomotor hyper-activity effects with

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repeated drug/environment pairings. In line with the presumed important role of dopamine in conditioning, morphine has been shown to increase dopamine activity in the ventral tegmental area (VTA) via the disinhibition of dopaminergic neurons in the VTA by the inhibitory action of morphine at the mu-opioid receptor on GABA inhibitory neurons (van der Kooy et al., 1982; Matthews and German, 1984; Johnson and North, 1992; Brown et al., 2012; Tan et al., 2012). In that the VTA is implicated in reward and that reward effects facilitate associative processes; it is not surprising that morphine stimulant effects are context specific (Leite Júnior et al., 2019; de Mello Bastos et al., 2020).

In the present study we measured the effects of morphine on extracellular signal regulated protein kinase activity (ERK) to assess the effects of morphine on activation of subcortical brain areas implicated in dopaminergic related reward effects, namely the VTA and nucleus accumbens (NAc). The rationale for measuring ERK in these brain structures was based on findings that have reported increases in ERK activity in dopaminergic projection areas (Adams and Sweatt, 2002; Girault et al., 2007; Lu et al., 2006; Radwanska et al., 2005) including the NAc (Borgkvist et al., 2008; Valjent et al., 2004; Kim and Kim, 2008; Fricks-Gleason and Marshall, 2011) following treatment with psychostimulant drugs such as cocaine. Additionally, in previous studies (Sanguedo et al., 2014, 2016, 2017, 2019), we showed that apomorphine induced context specific behavioral stimulation was associated with a strong ERK signal in brain dopamine systems. In the present study we used ERK as an indicator of activation in subcortical brain areas associated with the dopamine reward systems, namely the VTA and NAc.

In several recent reports we have also shown that morphine can induce locomotor stimulant effects (de Mello Bastos et al., 2020; Leite Júnior et al., 2019). In these studies, we administered morphine (10 mg/kg) immediately prior to placement in an open field arena for 30 minute test sessions and, consistently we observed that the locomotor stimulant effects were manifested during the second test session. More recently, Dias et al. (2021) using this same protocol, measured the locomotor response in 5 minute blocks. Interestingly, we found that while no locomotor stimulation was present on day 1, on subsequent test sessions when locomotor stimulation emerged, it occurred first in the initial 5 minute block of the 30 minute test session. In order to better assess this rapid onset of the locomotor stimulant response, we performed a time course experiment in separate groups of rats that were given acute 10 mg/kg morphine injections and then we measured ERK activation in VTA and NAc encephalic areas implicated in reward processes and found that peak ERK activation occurred at 5 minute post injection and declined substantially by 15 minute post-test and was absent at 30 minute post-test. In that we observed that both ERK activation in reward associated encephalic areas and locomotor stimulant effects of morphine occur in the initial 5 minute of a 30 minute test session, it seemed that a 5 minute test session should be sufficient to induce the locomotor stimulant effects. To directly examine this possibility, we administered morphine immediately pretest and limited the test session durations to 5 minute. The present report details the effects of this test protocol on morphine induced locomotor stimulation and ERK activation.

2. Methods

2.1. Subjects

Male Wistar albino rats provided by the State University of North Fluminense Darcy Ribeiro, initially weighing 200–300 g, were housed in individual plastic cages (25 × 18 × 17 cm) until the end of the experiment. Food and water were always available. The vivarium was maintained at a constant temperature (22 ± 2 °C), and a 12 h/12 h light/dark cycle (lights on at 07:00 h and off at 19:00 h). All experiments occurred between 8:00 and 12:00 h. For 7 days prior to all experimental procedures, each animal was weighed and handled daily for 5 minute. This process included being placed in a transport cage and taken to the injection administration bench located in an anteroom adjacent to the experimental testing room. The animals were given vehicle (VEH) injections. In this way the animals were familiarized with the handling and injection aspects of the testing protocol. The experimental procedures and the number of animals used in experiments were in accordance with the recommendations of the Commission of Ethics in Animal Experimentation (CEUA) of the University of North Fluminense Darcy Ribeiro-UENF (process 395/2018). These recommendations are in agreement with the ethical principles of animal research adopted by the Brazilian College of Animal Experimentation (COBEA) and by the National Council for Animal Experimentation Control (CONCEA).

2.2. Drugs

Morphine sulfate (Cristalia, São Paulo, Brazil) was used from 10 mg ampoules (1 ml) and was injected subcutaneously in the nape of the neck at a dose of 10 mg/kg. A 0.9% saline solution was used as vehicle. All doses were administered in a volume of 1.0 ml/kg body weight. Drug solutions were freshly prepared before each experiment.

2.3. Apparatus and behavioral measurements

The behavioral measurements were conducted in a black open field chamber (60 × 60 × 45 cm). A closed-circuit camera (IKEGAMI, model ICD-49) mounted 60 cm above the arena was used to record behavioral data. Locomotion, measured as distance traveled (m), was automatically analyzed using software EthoVision (Noldus, the Netherlands). The complete test procedure was conducted automatically without the presence of the experimenter in the test room. All behavioral testing was conducted under dim red light that favors exploratory behavior and avoids the possible aversive quality of white light (Nasello et al., 1998) as well as to enhance the contrast between the white subject and the dark background of the test chamber and reduce the animal's shadow.

2.4. Experimental procedure

Two test phases were conducted: acute and chronic. In line with our recent report (Dias et al., 2021) all subjects were first acclimated to the test arena by being injected with vehicle (VEH) and placed in the arena for 30 minute on 3 successive days. Using the locomotion distance traveled scores on the third habituation session, groups were formed equated for distance scores.

In the acute test phase, two groups ($n = 6$) were used. The groups were given either morphine (MOR-1 group) or vehicle (VEH-1group) immediately prior to placement in the test arena for 5 minute and immediately upon completion of their initial test session were euthanized and ERK measurements were performed in the VTA and NAc.

In the chronic test phase, there were five treatment groups: two vehicle groups ($n = 6$ each group), two paired groups (n = 6 each group) and an unpaired group (n = 6). In the paired groups (MOR—P), rats received morphine 10.0 mg/kg immediately before being placed into the arena test and vehicle administration 15 minute after removal from the arena test. In the unpaired group (MOR-UP), rats received vehicle immediately before being placed into the arena test and morphine 10.0 mg/kg 15 minute after being removed from the test environment to control for non-context associated MOR effects. The vehicle groups (VEH) received vehicle before being placed in the arena and 15 minute after removal from the arena. Treatments were administered on 5 consecutive days, one 5 minute session per day and this was denominated as the induction treatment phase. Day 6 was the conditioning test session in which one of the paired morphine groups received vehicle (MOR-P/VEH-6) and the other paired morphine group received morphine (MOR-P/MOR-6). The same procedure was followed with vehicle groups in which one vehicle group continued to receive vehicle (VEH/VEH-6) and the other vehicle group received morphine (VEH/MOR-6). The MOR-UP group received vehicle (MOR-UP/VEH-6). The animals received their treatments immediately before being placed into

the test environment for 5 minute. Immediately after completion of the sixth test session each animal in each group was euthanized and ERK measurements were performed on VTA and NAc tissue samples as described below.

2.5. Immunohistochemistry

The immunohistochemistry protocol was conducted as previously described by Sanguedo et al. (2014, 2016, 2017, 2019), which was adapted from that described by Marin et al. (2009). In brief, rats were rapidly anaesthetized by intraperitoneal injection of urethane (15 ml/kg; Sigma Aldrich, Saint Louis, MO, USA) prior to intracardiac perfusion of 4% paraformaldehyde (500 ml) in 0.1 M sodium phosphate buffer (pH 7.4) for 30 minute. The brains were removed and post-fixed for 2 h in 4% paraformaldehyde solution before transferring to 20% sucrose in 0.1 M sodium phosphate buffer (pH 7.4) for 48 h at 4 °C. Each brain was placed on an aluminum paper base and cryoprotected by a solution of water soluble glycols and resins (Tissue Tek® O. C. T. Sakura Finetek®, USA). The brain tissue was then frozen and maintained in liquid nitrogen until being processed for immunohistochemistry.

The encephalic areas sampled for the immunohistochemical analysis were ventral tegmental area (approximately −4.80 mm to −5.04 mm from bregma) and nucleus accumbens (core and shell approximately +2.52 mm to +2.76 mm from bregma). The coordinates adopted as the reference were obtained from Paxinos and Watson (2005). Four slices were collected sequentially from each brain structure of each animal and sectioned in a cryostat (Zeiss, Germany) at a thickness of 30 μm. The sections were placed onto previously gelatinized glass microscope slides to allow fixation. For immunohistochemistry, sections were rinsed three times for 10 minute in phosphate-buffered saline (PBS) and placed in blocking buffer (3% normal goat serum and 0.25% Triton X-100 in PBS) for 1 h at 22 °C. The sections were then treated with a freshly prepared solution of hydrogen peroxide (3% vol/vol) in chilled absolute methanol for 10 minute at 22 °C. Sections were then incubated for 24 h at 4 °C in 1:500 dilution of anti-phosphorylated-ERK antibody diluted in blocking buffer (Cat # 9101, Cell Signaling Technology®, Boston, MA, USA) as previously described. After the end of the incubation time, sections were washed 3 times for 10 minute each in PBS and incubated at 22 °C with a 1:100 dilution of biotinylated goat anti-rabbit IgG secondary antibody (BA-1000, Vector Laboratories®, CA, USA) in 1% normal goat serum and 0.25% Triton X-100 in PBS. Sections were then washed three times for 10 minute in PBS and processed using an ABC Elite kit (Vector Laboratories ®, Burlingame, CA, USA). In the next step, sections were washed again in PBS and processed with a DAB substrate kit for peroxidase (SK-4100, Vector Laboratories®, Burlingame, CA, USA) and incubated in DAB substrate simultaneously and timed precisely at 22 °C for 3 minute for color development of signal intensity. After drying, the slides were mounted with DPX (Sigma ®, USA).

Photomicrographs of brain sections were obtained using a CCD camera (Nikon Photometrics Cool Snap) attached to a light microscope (Nikon 80i, USA). Digital images were obtained at low magnification (10×) from regions of the brain identified from the Paxinos and Watson (2005) rat brain atlas and the labeled nuclei from each brain structure of each animal were quantified using the Image J® software “multi-point” tool. The number of labeled neurons was counted bilaterally from four sections and the average count of labeled neurons for these sections was used as the score for each animal. The number of p-ERK-labeled cells was normalized to the area of the quadrant counted. In order to assess details of the ERK-labeled cells, specific sections were photographed at higher magnification (40×).

Negative control slices were incubated with normal serum instead of the primary antibody (data not shown). To reduce any potential bias in the scoring, two experimenters unaware of the treatment groups independently performed the labeled nuclei counts. The counts obtained by each experimenter for each encephalic area and for each group were compared using Student's *t*-tests and no statistically significant differences ($p > 0.05$) were found between groups.

2.6. Statistics

For the morphine acute effects on locomotor activity and ERK activation, an independent t-test was used to compare vehicle versus morphine acute treatment. For the induction phase of the chronic test phase, a repeated two-way analysis of variance (ANOVA) was used to determine the group effect, day effect, as well as the interactions between both factors. When a significant effect of interaction was recorded, data were further statistically evaluated by one-way ANOVA followed by Tukey's test with $p < 0.05$ as the criterion for statistical significance. For the 5 minute total test session during the final test, a one-way ANOVA followed by Tukey's post-hoc tests using $p < 0.05$ as the criterion for statistical significance. For the within-treatment assessment of the behavioral activity during the final test, the total activity score was divided into 2 intervals of 5 minute each and a repeated measure two-way ANOVA was used to determine the treatment, intervals, and interaction between factors. When a significant effect of treatment group and intervals was obtained, data were further evaluated by one-way ANOVA followed by Tukey's post-hoc tests using $p < 0.05$ as the criterion for statistical significance. For morphine induced ERK activation, the findings were evaluated using a one-way ANOVA followed by Tukey's post-hoc tests with $p < 0.05$ as the criterion for statistical significance.

3. Results

3.1. Acute test phase

Fig. 1 shows the locomotion distance results and the number of phosphor ERK immunoreactive nuclei in the VTA and NAc for the MOR-1 and VEH-1 groups in the acute test phase. For the locomotion scores (Fig. 1A), an independent *t*-test showed that there was no difference between the VEH-1 and MOR-1 groups [$t(10) = 1.32$; $p > 0.05$]. Fig. 1B-C show the number of phosphor ERK immunoreactive nuclei. For the VTA (Fig. 1B), an independent *t*-test [$t(10) = 17.30$; $p < 0.01$; showed that MOR-1 had a higher number of phosphor ERK immunoreactive nuclei than the VEH-1 group. For the nucleus accumbens (Fig. 1C), an independent t-test showed that MOR-1 had a higher number of phosphor ERK immunoreactive nuclei than the VEH-1 group [$t(10) = 10.40$; $p < 0.01$;]. Fig. 1D-G shows examples of the sections used for counting phosphorylated-ERK-immunoreactive cells in the VTA and NAc following VEH and MOR acute administration.

3.2. Chronic test phase

Fig. 2 shows locomotor activity in distance scores over the course of the induction phase from day 1–5. For the 5 minute total test session (Fig. 1A), a repeated measures two-way ANOVA showed groups X days interaction [$F(8, 108) = 12.0$; $p < 0.01$], an effect of groups [$F(2, 27) = 20.40$; $p < 0.01$] and an effect of days of treatment [$F(4, 108) = 3.12$; $p < 0.05$]. A one-way ANOVA followed by Tukey tests to further analyze the interaction of group x days showed that on day 1, there was no difference among the groups [$F(4, 37) = 0.24$; $p > 0.05$]. On day 2 [$F(2, 27) = 10.0$; $p < 0.01$], day 3[$F(2, 27) = 32.33$; $p < 0.01$], day 4 [$F(2, 27) = 39.13$; $p < 0.01$] and day 5 [$F(2, 27) = 28.0$; $p < 0.01$], the MOR-P group had higher locomotion than all other groups ($p > 0.05$). In that the ERK immunolabeling was obtained at the end of the 5 minute test session, it is likely that the ERK effects only occurred in the latter part of the 5 minute test session and that this impact on behavior was missed. In consideration of this possibility, the analysis of the test session was subdivided into two 2.5 minute intervals. Fig. 1B shows the locomotor activity of the 2.5 minute intervals over the course of the induction phase for the initial 0–2.5 minute of each session. A repeated two-way ANOVA showed an interaction groups x days [$F(8, 108) = 7.0$; $p <$

MORPHINE ACUTE TEST PHASE

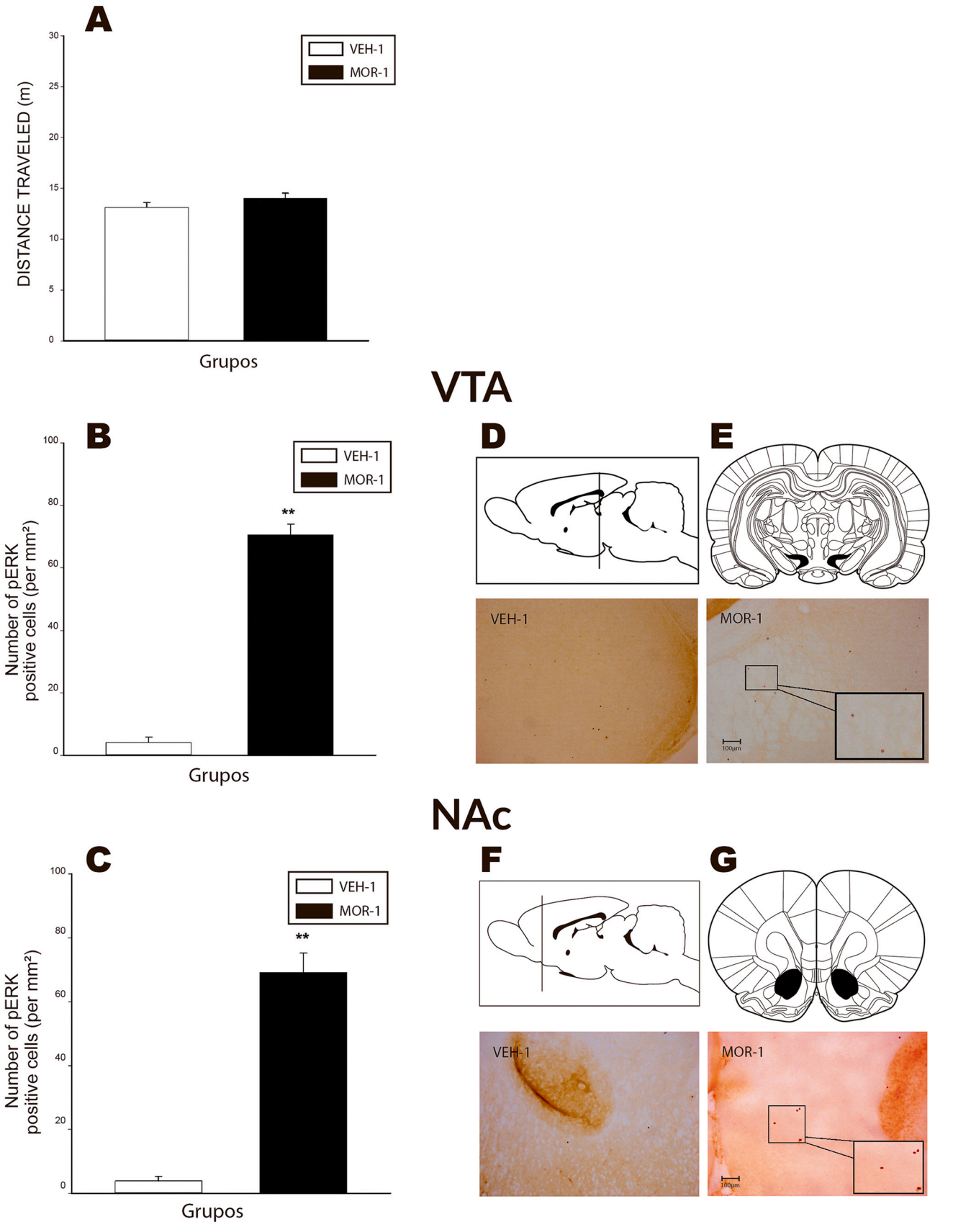

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Fig. 1. Acute test phase. **A:** Means and S. E. M. of locomotor activity during the 5 minute total test session with morphine 10 mg/kg and vehicle. **B** and **C:** Quantification of immunohistochemical results for ERK phosphorylation in the VTA (B) and NAc (C) following morphine 10.0 mg/kg and vehicle treatments. Data represent the mean ± S.E.M. ** ($p < 0.01$) denotes higher numbers of immunoreactive nuclei in the MOR-1 group when compared to the VEH-1 group (independent *t*-test). **D-G:** Drawings of sagittal section (E and G) displaying, at the vertical black bar, the location of coronal section (D and F), obtained from the Paxinos and Watson atlas (Paxinos and Watson, 2005). Representative photomicrographs of low (10×) and high (40× inserts) magnification images of ERK-P-immunoreactive cells from the VTA and NAc after VEH and MOR administration for the five minute activity test experiment. Scale bar indicates 100 μm in the low magnification images.

0.01], an effect of groups [F (2, 27) = 27.0; $p < 0.01$] but no effect of days of treatment [F (4, 108) = 0.8; $p > 0.05$]. A one-way ANOVA followed by the Tukey test to further analyze the interaction of group x days showed that on day 1, there was no difference among the groups [F (4, 37) = 0.41; $p > 0.05$]. On day 2 [F (2, 27) = 13.32; $p < 0.01$], day 3[F (2, 27) = 30.44; p < 0.01], day 4 [F (2, 27) = 31.51; $p < 0.01$] and day 5 [F (2, 27) = 27.20; p < 0.01], the MOR-P group had higher locomotion than all other groups (p > 0.05). Fig. 1C presents the distance scores in the 2.5–5.0 minute of the 5 test sessions. A repeated measures two-way ANOVA showed an interaction groups x days [F (8, 108) = 8.54; $p < 0.01$], an effect of groups [F (2, 27) = 12.83; $p < 0.01$] and an effect of days of treatment [F (4, 108) = 5.0; $p < 0.01$]. A one-way ANOVA followed by Tukey tests to further analyze the interaction of group x days showed that on day 1, there was no difference among the groups [F (4, 37) = 0.40; $p > 0.05$]. However, on day 2 [F (2, 27) = 6.0; $p < 0.01$], day 3 [F (2, 27) = 16.71; p < 0.01], day 4 [F (2, 27) = 20.81; p < 0.01] and day 5 [F (2, 27) = 21.20; $p < 0.01$], the MOR-P group had higher locomotion than all the other groups.

Fig. 3 shows the locomotor distance scores on the final test. For the 5 minute total test session (Fig. 3A), a one-way ANOVA showed that there was a difference among the groups [F (4, 25) = 30.81; $p < 0.01$] and the Tukey test showed that the MOR-P/VEH and MOR-P/MOR groups had higher locomotion than the other groups ($p < 0.05$). Fig. 3B shows the locomotor distance scores during the 2.5 minute intervals of the final test. A repeated measures two-way ANOVA showed only an effect of groups [F (4, 25) = 30.81; p < 0.01]. There was no effect of intervals [F (1, 25) = 0.02; $p > 0.05$] and no interaction groups x intervals [F (4, 25) = 1.0; p > 0.05]. The Tukey test showed that the MOR-P/MOR and MOR-P/VEH groups had higher locomotion than all other groups (p > 0.05).

Fig. 4 shows the number of phosphor ERK immunoreactive nuclei in the VTA and NAc. The ventral tegmental area results are presented in Fig. 4A and the one-way ANOVA showed that there was a difference among the groups [F (4, 25) = 64.61; $p < 0.01$] and the Tukey test showed that the MOR-P/VEH-6 and MOR-P/MOR-6 groups had a higher number of phosphor ERK immunoreactive nuclei than the VEH/VEH-6, VEH/MOR-6 and MOR-UP/VEH-6 groups ($p < 0.05$). For the nucleus accumbens (Fig. 4B), a one-way ANOVA showed that there was a difference among the groups [F (4, 25) = 53.40; p < 0.01;], and the Tukey test showed that the MOR-P/VEH-6 and MOR-P/MOR-6 groups had a higher number of phosphor ERK immunoreactive nuclei than the VEH/VEH-6, VEH/MOR-6 and MOR-UP/VEH-6 groups (p < 0.05). Fig. 1C-D show examples of the sections used for counting phosphorylated-ERK-immunoreactive cells in the VTA and NAc following VEH and MOR chronic administration.

4. Discussion

In this study we used a somewhat unconventional drug/behavior protocol in which we investigated the onset of morphine behavioral effects expressed as locomotor activity during the initial five minutes following a subcutaneous injection of morphine. We used this protocol to assess both the acute and chronic effects of the morphine treatment. The rational for this protocol was based on our earlier report (Dias et al., 2021) in which we found that subcutaneous injections of morphine (10 mg/kg) induced locomotor stimulant effects within the first 5 minute of a 30 minute test session. In the acute phase of the present study we found that the morphine treatment on day 1 had no effect upon locomotor activity compared with its vehicle control group. We did find however, that the acute morphine treatment on day 1 induced a substantial ERK activation in the VTA and NAc compared to the vehicle control group. The apparent behavioral neutrality of the initial morphine injection stands in contrast with a number of reports in which the initial 10 mg/kg morphine treatment induces a behavioral inhibition (Smith et al., 2009; Paul et al., 2021). One important difference between these studies is the time between the morphine injection and behavioral testing. In the present study and the other reports by our group (Dias et al., 2021; de Mello Bastos et al., 2020; Leite Júnior et al., 2019), in which the initial behavioral response did not differ from vehicle, is that the testing was initiated immediately after injection whereas in studies that found that morphine initially induced behavioral inhibition, testing was delayed for 15 to 30 minute post injection. Previously, we found increased ERK activation in VTA 5 minute post injection but not at 15 and 30 minute post injection. Thus, the role of VTA activation in the present report does not apply to the findings with 15 and 30 minute injection/testing delays. Also, the sensitization reports in the injection/delayed testing studies, in part, are attributable to the recovery from or tolerance to the initial inhibitory effects of the morphine treatments. Clearly, it is of substantial importance in future studies to use the present protocol with different post-injection delays (e.g. 0, 15, 30 minute.) and assess effects on behavior and ERK.

In view of the virtual equivalence between the morphine and vehicle groups in terms of day 1 locomotor activity versus the profound day 1 differential effects on ERK between the morphine and control groups, it is apparent that the morphine ERK effect cannot be related to locomotor activity perse. In the chronic treatment phase, we found that morphine induced potent locomotor stimulant effects. In line with our previous reports (Dias et al., 2021; de Mello Bastos et al., 2020; Leite Júnior et al., 2019) and, consistent with the acute test results, the locomotor stimulant effects did not occur following the first injection but were robust from morphine sessions 2–5. Importantly, on the day 6 conditioning test, there were no statistically reliable differences in locomotor stimulation or ERK activation between the paired morphine groups administered either morphine or vehicle. Although the conditioned response is typically a fraction of the drug induced response, in the present study, the conditioned response was comparable to the drug induced response. Seemingly this equivalence between the drug induced response and conditioned response is related to the brevity of the 5 minute test session. In clinical studies of drug relapse, exposure to drug associated cues can elicit intense, albeit brief, drug like responses and initiate craving. By briefly acting like the drug, the cues can reactivate the drug state and initiate drug seeking (O'Brien et al., 1998; Crombag et al., 2008; Perry et al., 2014; Wheeler et al., 2015). In consideration of the importance of the conditioned drug response to relapse and the maintenance of addictive behavior, we have focused on brief test sessions, as a way to better assess the intensity of the conditioned response. In line with the intensity of the initial conditioned response to drug associated cues, the present findings indicate that indeed the drug contextual cues evoked a response that was as intense as the drug treatment itself. Furthermore, the activation of ERK in subcortical brain structures associated with dopamine and reward processes (Shiflett et al., 2008; Kirschmann et al., 2014) by contextual cues was comparable to the activation induced by morphine in morphine treated animals. Furthermore, the ERK activation induced by the conditioned morphine response was substantially greater than the acute morphine treatment in the VEH group given MOR on the final test. While it is unclear in the present data as to the contribution of

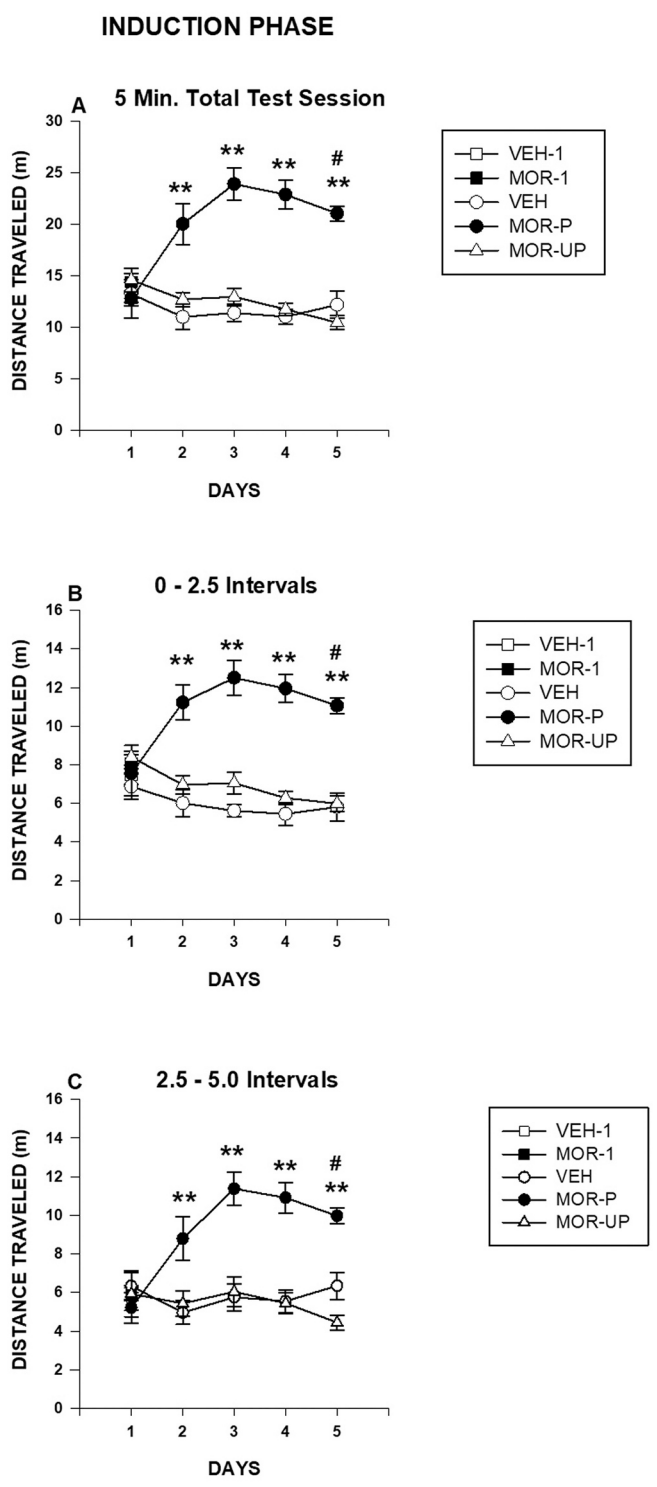

Fig. 2. Means and S. E. M. for the 5 minute total test session (A), first 2.5 minute interval (B) and last 2.5 minute interval (C) during the induction phase. ** denotes higher locomotion than the other groups; # Denotes a significant difference between the first and the last day of the induction phase for the same experimental group (repeated measures two-way ANOVA followed by Tukey's post hoc test).

the locomotor activation to the ERK effects, it needs to be noted that the acute morphine treatment following the first test session did induce an ERK response without an effect on locomotor activation that was comparable to the ERK response in the chronic MOR group that had a locomotor stimulant response. Also, the MOR day 1 ERK response was substantially greater than the ERK response in the VEH group given MOR on test day 6, even though neither group had elevated activity levels.

Morphine has been shown to have an indirect dopamine agonist effect (Matthews and German, 1984; Johnson and North, 1992; Matsui et al., 2014), by disinhibition of dopamine neuronal activity via mu-opioid receptor inhibition of inhibitory GABA neurons. Presumably, the increase in ERK activity reflects this disinhibitory activation in dopamine systems in encephalic reward structures such as the VTA that was elicited during the 1st test session. For this disinhibition effect to increase activity in dopamine systems there must be ongoing activity in the dopamine system to disinhibit. In this regard, morphine can act like an indirect dopamine agonist such as cocaine, in that it requires an intact active dopamine system to induce behavioral stimulation. Evidently, in the chronic phase of the present study in which an acute morphine treatment was administered following 5 VEH test sessions, the activity level of the dopamine neurons in the VTA had largely subsided by the sixth test session in that the morphine acute treatment on test session 6 did not have a statistically reliable effect upon ERK activity. In line with this consideration are the findings in our recent study (Ferreira et al., 2022) on the post-trial effects of 10 mg/kg of morphine on locomotor activity. We showed the post-trial morphine immediately following a 5 minute exposure to a novel environment, generated hyper-activity during the next exposure to the same environment. When the same morphine treatment was administered after the animals had received six 5 minute pre-exposures to the test environment, morphine had no effect on behavior dui the next exposure to the test environment. Critically, we showed that pre-exposures to the test environment induced a habituation acquisition function. In the present study, the first 5 minute test session was still a relatively novel event and that this novelty seemingly initially activated the reward systems in the VTA and NAc (Bardo et al., 1996; Rebec et al., 1997) that was enhanced by morphine. Evidently, this relative novelty effect had largely subsided by the sixth test session in that the morphine acute treatment on test session 6 did not have a statistically reliable effect upon ERK activity.

The conditioned behavioral stimulant response remains a conundrum in terms of Pavlovian conditioning. That is, when this locomotor conditioning is framed in terms of Pavlovian conditioning, the test arena is the conditioned stimulus (CS) and morphine is the unconditioned stimulus (UCS). In Pavlovian conditioning the UCS evokes an unconditioned response (UCR) and it is the UCR that becomes associated with the CS so that the CS then comes to elicit the UCR. In the present experiment the test arena, CS elicits the behavioral UCR on the second test even though there was no apparent behavioral UCR in the first session. On the other hand, the occurrence of ERK activation in the VTA and NAc seems to fit into a Pavlovian conditioning framework. That is, the unconditioned morphine UCR is ERK activation in the VTA and NAc and it is paired with the contextual open field arena CS so that the test environment acquires CS properties and comes to evoke the ERK activation as a CR. While this analysis provides a Pavlovian account of the conditioned ERK response, it does not appear to account for the conditioned behavioral stimulation. The behavioral stimulant effect, however, seems better considered as an indirect effect of activation in brain reward systems. Numerous studies have shown that increases/decreases in brain dopamine activity can have potent effects upon learning and memory by increasing/decreasing reward effects (Koob, 2009; Koob and Volkow, 2016), and by increasing/decreasing the incentive value of stimuli (e.g. Beninger, 1983; Mazurski and Beninger, 1991). The increase in the incentive value of associated cues promotes increased activity in rodents (Robinson and Berridge, 2001; Wyvell and Berridge, 2001). In the present study, morphine administered on day 1 induced a prominent activation of ERK and this ERK response became associated with the contextual cues. While morphine can enhance activity in reward associated brain systems (Kim et al., 2016) these reward systems do not directly activate behavior but rather enhance stimuli and responses associated with the activation in the reward systems. While the

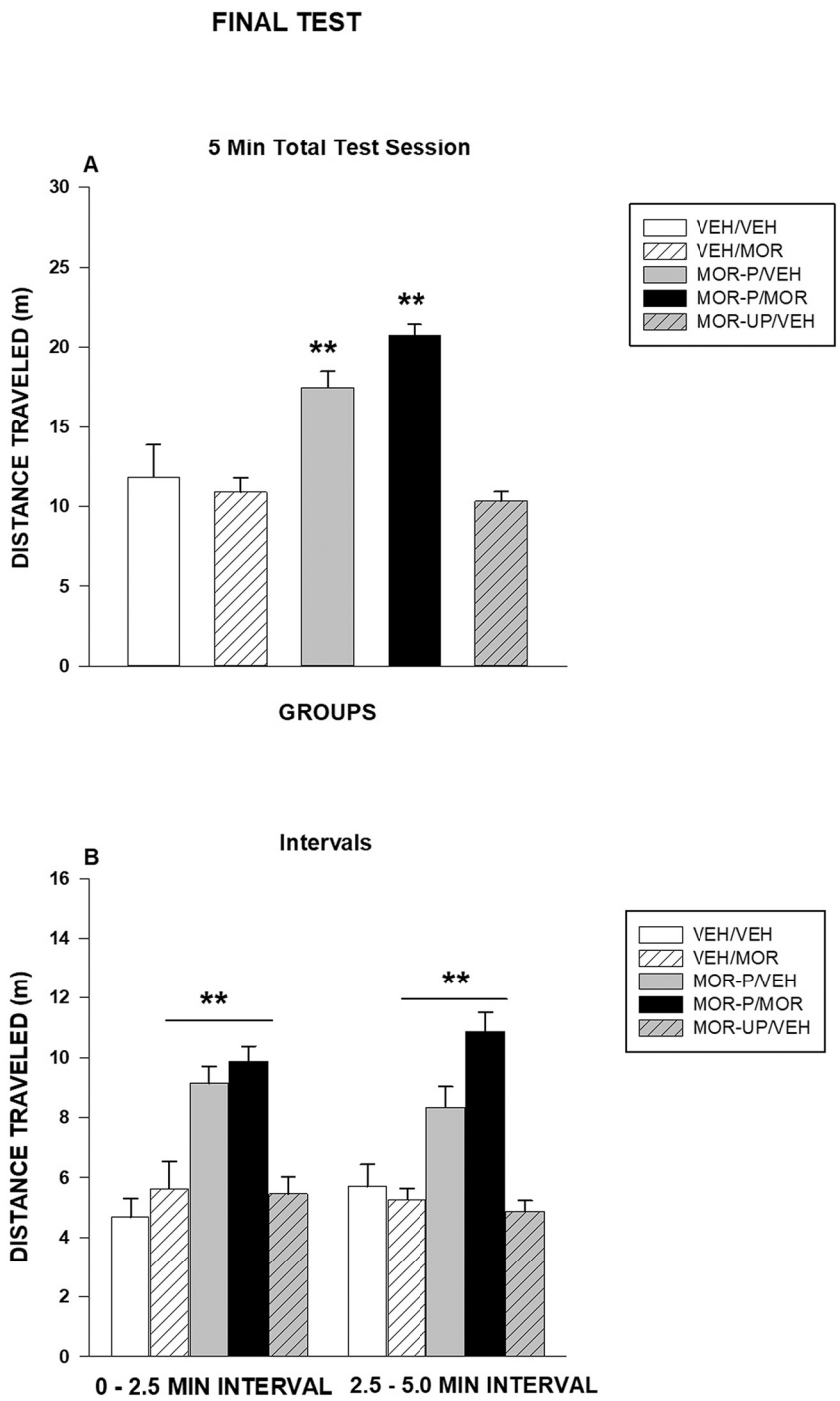

Fig. 3. Means and S. E. M. for the 5 minute total test session (A) and for two successive 2.5 minute intervals (B) during the final test on day 6. ** Denotes higher locomotion than the other groups (one-way ANOVA followed by Tukey's post hoc test).

importance of contiguity has long been established for a CS-CR association it had been assumed that it occurred immediately (Guthrie, 1935) but, following the seminal study by Duncan (1949), it has since been established that the association needs a consolidation period to be incorporated into memory. Presumably then, the association of the contextual cues with the morphine activation of brain reward systems was consolidated on day 1 and consequently only manifested in behavior on the second test session after the reward systems had become linked to the arena cues. In fact, we have shown that morphine administered immediately posttrial during memory consolidation (Ferreira et al., 2022) can evoke a behavioral stimulant effect one day later without morphine even though the morphine drug state was never experienced directly in the presence of the drug cues. Furthermore, in the present experiment on the day 2 test, the morphine locomotor stimulant response was robust in the initial 2.5 minute of the test session. This rapid onset of the locomotor stimulant response fits better with a contextual cue evoked response rather than a drug evoked response by the subcutaneous drug injection.

In conclusion, given the important impact of conditioned stimuli to addictive behavior such as morphine addiction, the use of a brief test session appears to offer a valuable methodology to investigate the initial response to a conditioned drug cue. In that brain reward systems are generally considered a key determinant of the addictive potency, we primarily directed our ERK focus on activation of the brain areas

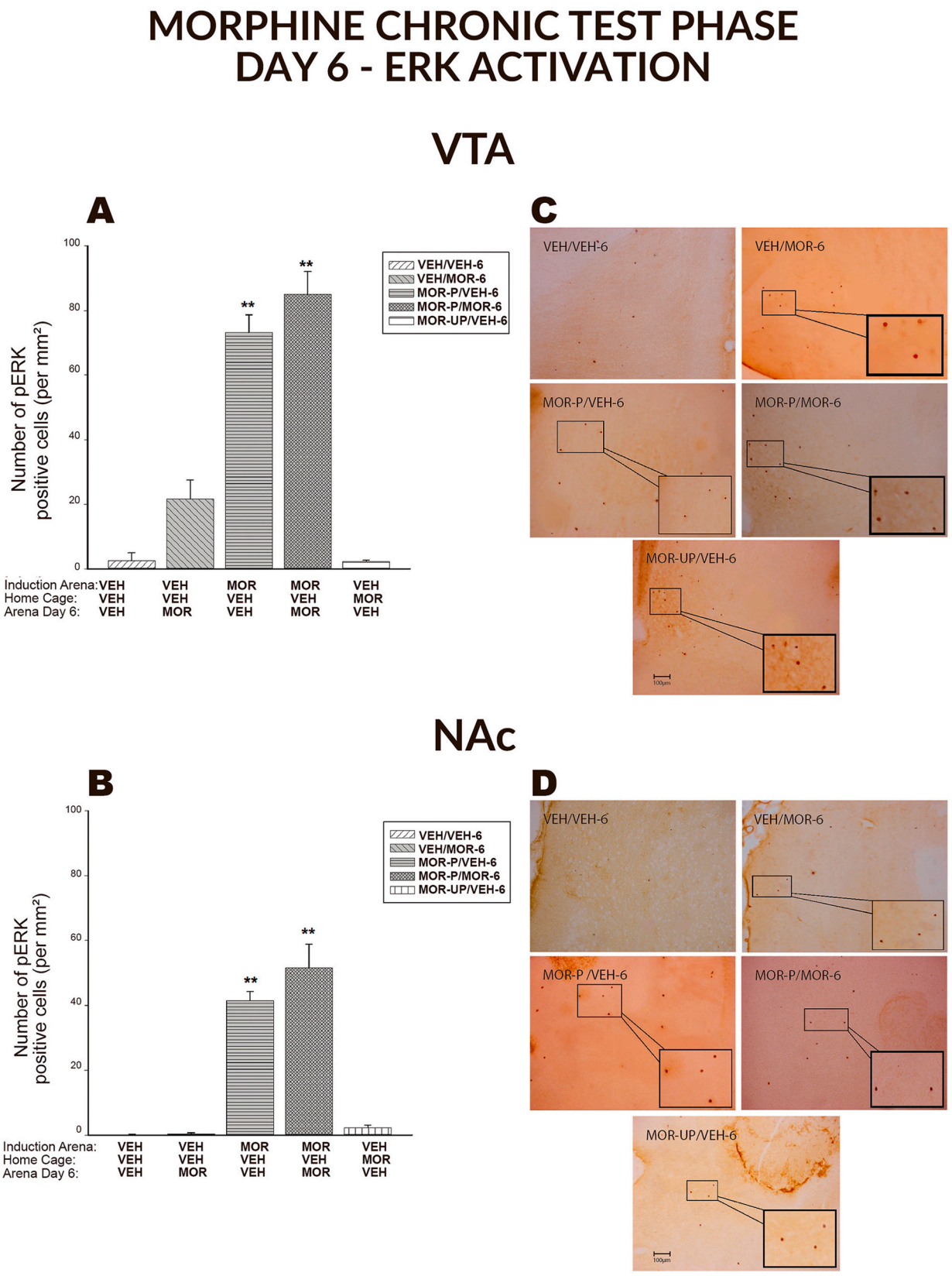

Fig. 4. Morphine chronic test phase day 6 - ERK activation. **A-B:** Quantification of immunohistochemical results for ERK phosphorylation in the VTA (A) and NAc (B) following chronic morphine (10.0 mg/kg) and vehicle treatments. Data represent the mean ± S.E.M. ** ($p < 0.01$) Denotes higher numbers of immunoreactive nuclei in the MOR-P groups when compared to the other groups (one-way ANOVA followed by the Tukey test). **C-D:** Representative photomicrographs of low (10×) and high (40× inserts) magnification images of ERK-P-immunoreactive cells from the VTA and NAc after VEH and MOR administration. Scale bar indicates 100 μm in the low magnification images.

considered important in the mediation of reward processes. Seemingly, it is the cue activation of brain reward systems that is most relevant to the maintenance of addiction as well as the initiation of relapse during abstinence. The findings in the present study suggest that the brief test procedure used in this investigation coupled with behavioral and neurobiological measures may be useful in assessing the efficacy of potential treatments to attenuate/eliminate opioid conditioning.

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