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Morphine reward effects and morphine behavioral sensitization: The adventitious association of morphine activation of brain reward effects with ongoing spontaneous activity

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ABSTRACT

The development of sensitization is one of the hallmarks of addictive drugs such as morphine. We administered morphine (10 mg/kg; MOR) to induce locomotor sensitization and ERK activation in the VTA and NAc. In the first experiment, four groups of rats received five daily 30 min sessions in an open-field, and locomotion was measured. For the first four sessions, one group received MOR pre-test (MOR-P); a second group received vehicle pre-test (MOR-UP) and MOR 30 min post-test; the remaining 2 groups received vehicle (VEH) pre-test. On the fifth session, the MOR-P, MOR-UP, and one VEH group received MOR pre-test and the remaining VEH group received VEH. Sensitization emerged in the first 5 min and progressed over to the second and third 5 min blocks only in the MOR-P group. For the second experiment, 4 groups received MOR and 4 groups VEH, and were then returned to their home cage and after 5, 15, 30 or 60 min post-injection, were euthanized for ERK measurements in VTA and NAc. ERK activation increased and peaked at 5 min post injection in the MOR group and then declined to VEH levels by 30 min. Another two groups received either MOR or VEH immediately before a 5 min arena test and ERK was measured immediately post-test. MOR had no effect on locomotion but increased ERK in the VTA and NAc. The peak ERK activation in VTA reflected activation of reward systems by morphine that reinforced locomotor behavior and with repeated treatments, induced a sensitization effect.

1. Introduction

It has frequently been demonstrated in preclinical behavioral pharmacology studies that repeated treatments with psychostimulant drugs potentiate the initial behavioral drug response. This behavioral sensitization has been considered to be a characteristic of addictive drugs. The use of drug-induced changes in spontaneous motor behavior as a dependent behavioral variable has been employed in numerous studies to assess stimulant behavior induced by several different drugs that have pro-dopamine effects such as amphetamine (Di Chiara and Imperato, 1988; Anagnostaras and Robinson, 1996), cocaine (Post et al., 1992; Carey and Gui, 1998; Vaher et al., 2020), apomorphine (Mattingly et al., 1988; Bloise et al., 2007) and morphine (Weisberg and Kaplan, 1999; Liu et al., 2014; de Mello Bastos et al., 2020). These studies in rodents have shown that drug induced locomotor hyper-activity effects undergo behavioral sensitization with repeated drug/environment pairings. Recently, we have also reported locomotor conditioning and sensitization effects in rats following repeated morphine (10.0 mg/kg) treatments (Leite Junior et al., 2019). In these experiments we measured the locomotor stimulant effects of morphine in 30 min test sessions. Interestingly, we found that the conditioned stimulant effects only occurred in the first 5 to 10 min of the 30 min conditioning test. Prompted by this

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observation we undertook in the present investigation to monitor the emergence of morphine locomotor sensitization induced by 10 mg/kg morphine within successive 5 min blocks over the course of successive 30 min test sessions.

In order to have a neurobiological effect of morphine, in addition to the behavioral response, we included the measurement of ERK. The extracellular signal regulated kinase (ERK) cascade is an important signaling pathway that underlies synaptic plasticity, cellular excitability, learning and arousal. Furthermore, psychostimulant drugs such as cocaine produce an increased ERK response in dopaminergic projection areas (Valjent et al., 2005; Fricks-Gleason and Marshall, 2011), including the nucleus accumbens (NAc) (Marin et al., 2009). In previous studies (Sanguedo et al., 2014, 2019) of apomorphine behavioral sensitization we showed that this sensitization was associated with increased ERK activity in brain dopamine systems. In the present investigation we measured ERK activity in key dopaminergic brain areas, which are important in the brain reward system, namely the ventral tegmental area (VTA) and nucleus accumbens (NAc). These dopaminergic subcortical brain areas are disinhibited by the inhibitory action of morphine at the μ receptor on GABA inhibitory neurons (Matthews and German, 1984; Matsui et al., 2014). Also, in our previous report (de Mello Bastos et al., 2020) we found that the MOR sensitization response of increased locomotor activity did not occur in the initial test session. In order to better evaluate this absence of behavioral activity evoked by MOR in the initial test session, we conducted an additional ERK experiment in which we measured MOR induced effects on ERK activity in the VTA and NAc immediately following the first 5 min of a test session.

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 freely available at all times. The vivarium was maintained at a constant temperature (22 ± 2 °C), and a 12/12 h light/dark cycle (lights on at 7 h and off at 19 h). All experiments occurred between 12 h and 17 h. For 7 days prior to the start of behavioral testing procedures, each animal was weighed and handled daily for 5 min. All experiments were performed in compliance with Brazilian Council for Animal Experimentation Control (CONCEA), which are based on the US National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

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 arena (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 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 to avoid the possible aversive quality of white light and to enhance the contrast between the white subject and the dark background of the test chamber. Nasello et al. (1998) have shown that red light conditions elicit exploratory behavior and not behaviors indicative of anxiety. A fan in the experimental room provided masking noise. The fan was turned on immediately prior to placing the animal in the experimental arena and turned off upon removal of the animal from the experimental arena.

2.4. Experimental procedures

In the first experiment, 4 groups (n = 7) were used. Initially all subjects were acclimated to the test arena by being tested for 30 min on 3 successive days. Immediately prior to each of the habituation test days, the subjects received VEH injections. Based on the locomotion distance scores for the third habituation session, 4 groups were formed and equated for distance scores. Next, the groups were given morphine (MOR; 10 mg/kg) or vehicle (VEH) injections immediately prior to being placed in the arena for 30 min test sessions. The 4 groups were given 30 min tests on 5 successive days. One group (MOR-P-5) received MOR immediately prior to each test session; a second group (VEH-5) was injected with VEH immediately before each test session; a third group (MOR-UP4/MOR-P1) used for the first 4 test sessions, was injected with VEH immediately at the start of the test session and at 30 min post-test, was given MOR in the home-cage (i.e., the unpaired morphine group) but, on the fifth test session was injected with MOR just prior to session 5. The final group (VEH-4/MOR-P1) received VEH injections prior to the first 4 tests but MOR before the fifth session.

For the second experiment, in order to assess the magnitude and time course of ERK induction by the morphine treatment (10.0 mg/kg) used in the present study, eight groups of rats were given either vehicle (4 groups, n = 4 for each group) or 10.0 mg/kg morphine (4 groups; n = 4 for each group) and returned to their home-cage and either 5, 15, 30 or 60 min later were euthanized and ERK measurements were made in the VTA and NAc.

A complementary ERK experiment (i. e., 5 min activity test) was conducted to measure the impact of morphine on ERK following locomotor activity during the first 5 min of a test session. The same habituation protocol was used as was used in experiment 1. The rats were initially acclimated to the test arena by being tested for 30 min on 3 successive days. Subsequently, the rats were subdivided into 2 groups (n = 5) and equated for distance scores during the final 30 min acclimation test. One group received MOR (10 mg/kg) and the other group VEH. Immediately following their respective MOR/VEH injection the rats were placed in the test arena for a 5 min test session. Following completion of the 5 min test, the rats were immediately euthanized, brains dissected and assayed for ERK as in experiment 1.

2.5. Immunohistochemistry

The immunohistochemistry protocol was conducted as previously described by Sanguedo et al. (2014, 2016, 2017, 2019) and 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 min. 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 two brain areas sampled for the immunohistochemical analysis were nucleus accumbens (core and shell approximately +2.52 mm to +2.76 mm from bregma) and ventral tegmental area (approximately −4.80 mm to −5.04 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 min 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 min 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 min 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 min 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 min 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-positive cells was normalized to the area of the quadrant counted. In order to demonstrate details of the ERK-positive cells, specific sections were photographed at higher magnification (40×).

Negative control slices were incubated with normal serum instead of primary antibody (data not shown). In order to minimize 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. Statistical analyses

For the pharmacological treatment phase of experiment 1, a repeated two-way analysis of variance (ANOVA) was used in order 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 within-treatment assessment of the behavioral activity, the total activity score was divided into 6 intervals of 5 min 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 the time course effect of morphine induced ERK activation, a two-way analysis of variance (ANOVA) was used to evaluate the group effect, interval effect, as well as the interaction between factors. When a significant effect of interaction was recorded, data were further statistically evaluated by an independent *t*-test or by one-way ANOVA followed by Tukey's post-hoc test with $p < 0.05$ as the criterion for statistical significance. For the 5 min activity test experiment, an independent t-test was used to evaluate both the locomotor activity and the number of phosphor ERK immunoreactive nuclei when comparing groups.

3. Results

Fig. 1 shows the locomotion during 5 days of the induction phase. For locomotion during the 5 induction days (Fig. 1A), according to a repeated two-way ANOVA, there was a significant effect of groups × days interaction [$F(12, 96) = 11.12$; $p < 0.01$], of groups [$F(3, 24) = 15.0$; $p < 0.01$], but no significant effect of days [$F(4, 96) = 1.0$; $p > 0.05$]. A one-way ANOVA followed by Tukey's post hoc test to further analyze the groups × days interaction, showed that neither on day 1 [$F(3, 24) = 1.04$; $p > 0.05$] nor on day 2 [$F(3, 24) = 1.33$; $p > 0.05$], were there any significant differences among the groups. However, on day 3 [$F(3, 24) = 7.0$; $p < 0.01$], day 4 [$F(3, 24) = 21.0$; $p < 0.01$] and day 5 [$F(3, 24) = 45.10$; $p < 0.01$], the MOR-P/MOR group had higher locomotion than all other groups. For the locomotor activity during the last day (day 5) of the induction phase (Fig. 1B), according to a one-way ANOVA, there was a significant difference between the groups [$F(3, 24) = 45.10$; $p < 0.01$] and the MOR-P/MOR group had higher locomotor activity than all the other groups (Tukey's post hoc test; $p < 0.05$).

Fig. 2 shows the within session locomotion for each day of the induction phase. For the within session locomotion, the 30 min locomotion session was divided into 6 intervals of 5 min each. For induction day 1 (Fig. 2A), according to a repeated two-way ANOVA, there was only a significant effect of intervals [$F(5, 120) = 75.90$; $p < 0.01$]. There was no significant effect of groups [$F(3, 24) = 1.04$; $p > 0.05$], neither a significant interaction of groups × intervals [$F(15, 120) = 1.11$; $p > 0.05$]. For induction day 2 (Fig. 2B), according to a repeated two-way ANOVA, there was a significant effect of the interaction between groups × intervals [$F(15, 120) = 2.43$; $p < 0.01$] and a significant effect of intervals [$F(5, 120) = 68.70$; $p < 0.01$] but no significant effect of groups [$F(3, 24) = 1.33$; $p > 0.05$]. For induction day 3 (Fig. 2C), according to a repeated two-way ANOVA, there were significant effects of groups × intervals interaction [$F(15, 120) = 2.0$; $p < 0.01$], of groups [$F(3, 24) = 7.0$; $p < 0.01$] and of intervals [$F(5, 120) = 92.60$; $p < 0.01$]. For intervals 1 [$F(3, 24) = 5.75$; $p < 0.01$] and 2 [$F(3, 24) = 8.50$; $p < 0.01$], the MOR-P5 group had higher locomotion than all the other groups (one-way ANOVA followed by Tukey's post hoc test; $p < 0.05$). For intervals 3–6, there was no difference among the groups. For induction day 4 (Fig. 2D), according to a repeated two-way ANOVA, there were significant effects of groups × days interaction [$F(15, 120) = 7.0$; $p < 0.01$], groups [$F(3, 24) = 20.80$; $p < 0.01$] and of intervals [$F(5, 120) = 47.40$; $p < 0.01$]. Considering intervals 1 [$F(3, 24) = 23.0$; $p < 0.01$] and 2 [$F(3, 24) = 10.34$; $p < 0.01$], the MOR-P5 group had higher locomotion than all the other groups (one-way ANOVA followed by Tukey's post hoc test; $p < 0.05$). During intervals 3–6, there was no difference among the groups ($p > 0.05$). For induction day 5 (Fig. 2E), according to a repeated two-way ANOVA, there were significant effects of groups × intervals interaction [$F(15, 120) = 5.82$; $p < 0.01$], of groups [$F(3, 24) = 45.07$; $p < 0.01$] and of intervals [$F(5, 120) = 72.02$; $p < 0.01$]. A one-way ANOVA followed by Tukey's test showed that for interval 1 [$F(3, 24) = 25.50$; $p < 0.01$], the MOR-P5 group had higher locomotion than all the other groups ($p < 0.05$) and the VEH-4/MOR-P1 and MOR-UP4/MOR-P1 groups had higher locomotion than the VEH-5 group ($p < 0.05$). For interval 2 [$F(3, 24) = 17.11$; $p < 0.01$] and interval 3 [$F(3, 24) = 12.50$; $p < 0.01$], the MOR-P5 group had higher locomotion than all the other groups ($p < 0.05$). For intervals 4 through 6, there was no significant difference among groups ($p > 0.05$).

Fig. 3 shows the number of phosphor ERK immunoreactive nuclei for the ERK home cage time course curve produced by morphine. For the VTA (Fig. 3A), according to a two-way ANOVA, there were significant effects of groups × time interaction [$F(3,32) = 15.21$; $p < 0.01$], of groups [$F(1,32) = 50.50$; $p < 0.01$] and of time [$F(3, 32) = 17.0$; $p < 0.01$]. To further analyze the interaction of group × time, independent *t*-tests were performed comparing VEH and MOR groups. These tests confirmed that the differences at 5 and 15 min post injection were highly

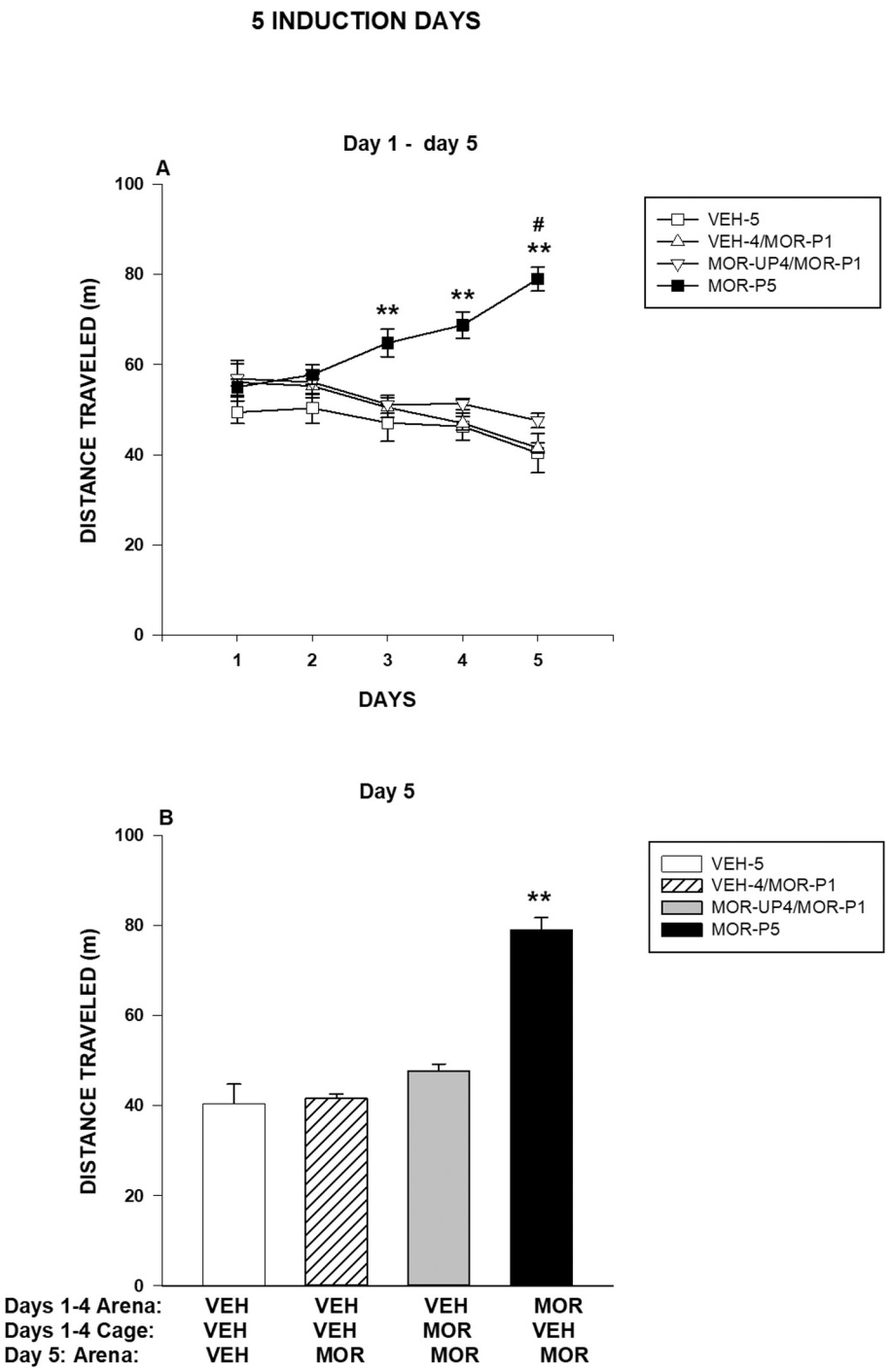

Fig. 1. Means and S. E. M. of locomotor activity during pharmacological treatment with morphine 10 mg/kg and vehicle. ** denotes significantly higher locomotor activity than all the other groups. # denotes that for the morphine paired group, the locomotor activity on the 5th day was significantly higher than on the 1st day ($p < 0.05$; repeated ANOVA followed by Tukey's test).

significant statistically at 5 [$t(6) = 6.0$; $p < 0.01$] and 15 min [$t(6) = 4.70$; $p < 0.01$], the morphine group had a higher number of phosphor ERK immune-reactive nuclei than the vehicle group. For the time course of ERK phosphorylation after VEH injections, a one-way ANOVA showed that there was no difference between times after injections [$F(3,12) = 0.30$; $p > 0.05$]. However, for the MOR treatments, there was a difference between times after injections [$F(3, 12) = 25.20$; $p < 0.01$] and Tukey test showed that for 5 and 15 min after MOR injections, there were a higher number of phosphor ERK immune-reactive nuclei than at 30 and 60 min ($p < 0.05$).

For the NAc (Fig. 3B), according to a two-way ANOVA, there were significant effects of group × time interactions [$F(3, 32) = 15.50$; $p < 0.01$], of groups [$F(1, 32) = 46.12$; $p < 0.01$], and of time [$F(3, 32) = 23.30$; $p < 0.01$]. According to an independent *t*-test to further analyze the interaction of group × time post injection at 5 min [$t(6) = 5.93$; $p < 0.01$] and 15 min [$t(6) = 3.84$; $p < 0.01$], the morphine group had a higher number of phosphor ERK immune-reactive nuclei than the vehicle group. For the time-course of ERK phosphorylation after VEH injections, a one-way ANOVA showed that there was no difference for time after injections [$F(3, 12) = 2.92$; $p > 0.05$]. However, for the MOR

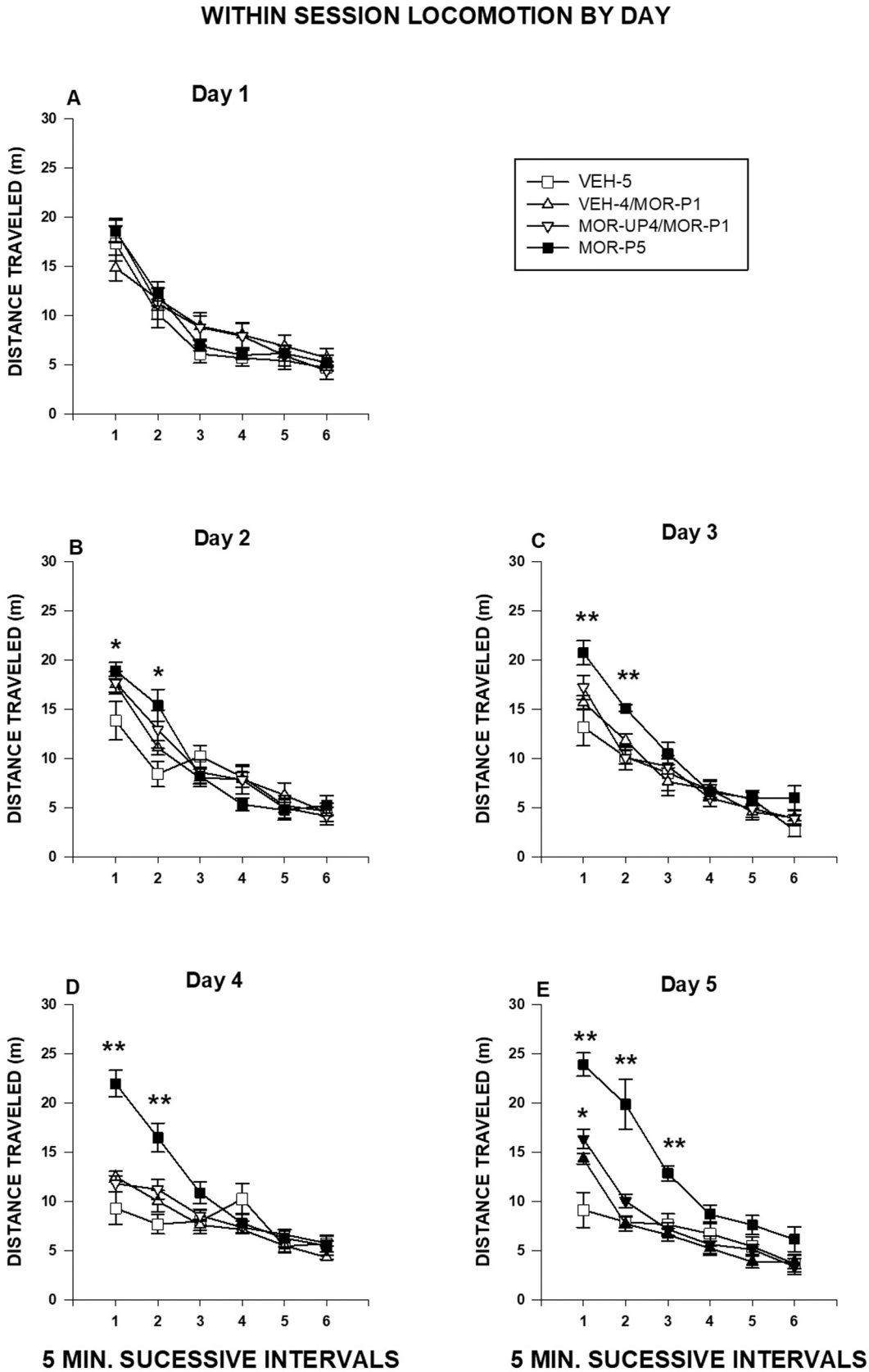

Fig. 2. Means and S. E. M.s of within group comparisons for locomotor distance scores. ** denotes higher locomotor activity than all the other groups. * denotes higher locomotor activity than the vehicle group ($p < 0.05$; repeated ANOVA followed by Tukey's test).

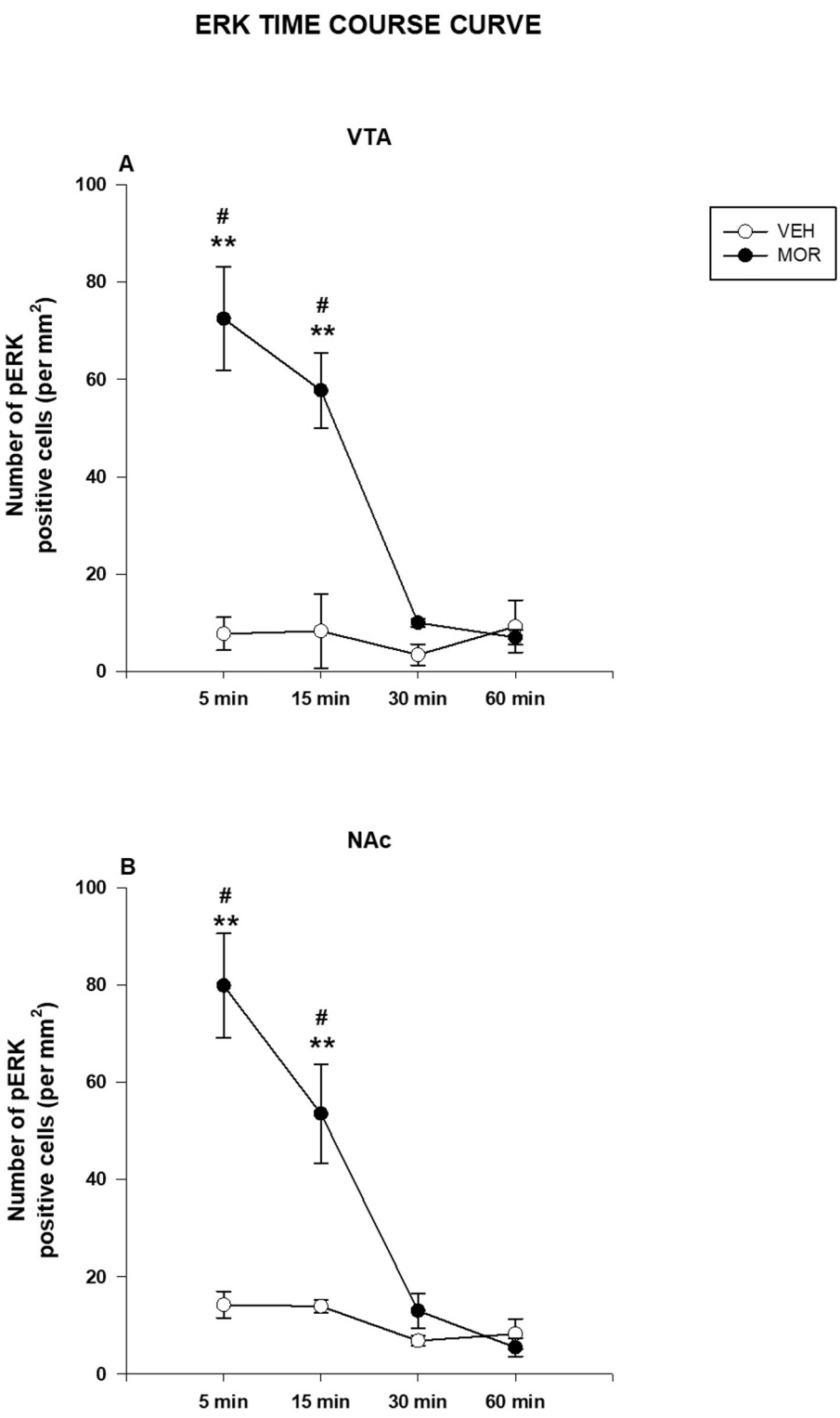

Fig. 3. Quantification of immunohistochemical results for ERK phosphorylation in the VTA (A) and NAc (B) following morphine 10.0 mg/kg and vehicle injections at 5, 15, 30 and 60 min. Data represents the mean ± S.E.M. ** ($p < 0.01$) denotes higher numbers of immunoreactive nuclei in the MOR group when compared to the VEH group in the same brain area. * ($p < 0.05$) denotes higher numbers of immunoreactive nuclei in the MOR group when compared to the VEH group in the same brain area (two-way ANOVA and independent *t*-test).

treatments, there was a difference between times after injections [$F(3, 12) = 20.80$; $p < 0.01$] and Tukey test showed that for 5 and 15 min after MOR injections, there were a higher number of phosphor ERK immunereactive nuclei than at 30 and 60 min ($p < 0.05$).

Figs. 4 and 5 show examples of the microscope sections used for counting phosphorylated-ERK-immunoreactive cells in the VTA (Fig. 4) and nucleus accumbens (Fig. 5), corresponding to the groups at 5, 15, 30 and 60 min after treatment administration.

Fig. 6 shows the results of the locomotion and ERK in the MOR and VEH groups after the 5 min activity test. For the locomotion scores (Fig. 6A), an independent *t*-test showed that there was no effect of groups [$t(8) = 0.50$; $p > 0.05$]. Fig. 6B–C show the number of phosphor ERK immunoreactive nuclei. For the VTA (Fig. 6B), an independent t-test showed that there was an effect of groups [$t(7) = 2.63$; $p < 0.05$] and that the MOR group had a higher number of phosphor ERK immunereactive nuclei than the VEH group. For the NAc (Fig. 6C), an independent t-test showed that there was an effect of groups [$t(7) = 4.80$; $p < 0.01$] and that the MOR group had a higher number of phosphor ERK

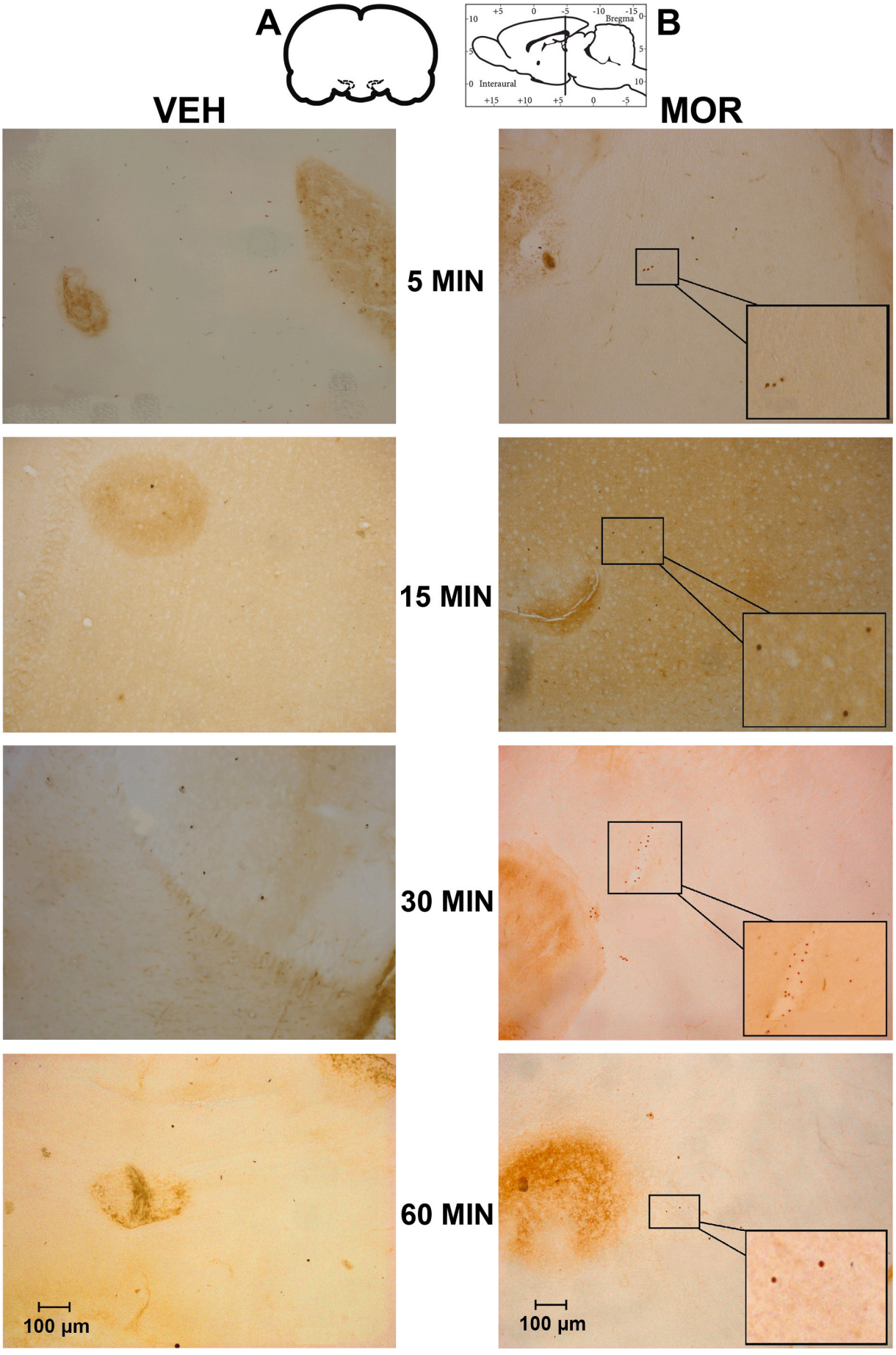

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Fig. 4. Representative low (10×) and high (40× inserts) magnification images of ERK-P-immunoreactive cells from the VTA 5, 15, 30 and 60 min after VEH and MOR administration for the time course experiment. Drawings of Sagittal section (B) displaying, at the vertical black bar, the location of coronal section (A) were obtained from the Paxinos and Watson atlas (2005). Scale bar indicates 100 μm in the low magnification images.

immune-reactive nuclei than the VEH group. Fig. 6D shows examples of the sections used for counting phosphorylated-ERK-immunoreactive cells in the VTA and NAc following VEH and MOR administration.

4. Discussion

In line with previous studies (Vezina and Stewart, 1984; Neisewander and Bardo, 1987; Powell and Holtzman, 2001; Lu et al., 2002; Sharf et al., 2010), we found that repeated morphine treatments in rats induced a behavioral locomotor sensitization effect. This effect was context selective in that similar morphine treatments administered unpaired to the test arena did not result in locomotor stimulant effects. What was new and interesting in the present study is that we found that when we subdivided the 30 min activity sessions into successive 5 min blocks, the behavioral sensitization only occurred in the first three 5 min blocks and that there was no effect on activity in the three subsequent sessions. Furthermore, the overall increases in activity only began to emerge after the second test session and initially in the first 5 min block of that test session. In that the testing was initiated immediately after the morphine injection, this rapid behavioral response to the injection indicated that the drug effect was fast acting and seemingly brief in duration. In our follow up experiment, in which we measured the ERK response in the VTA and NAc at 5 post-injection time points, we found that the morphine injections markedly increased ERK activity that peaked at 5 min. This transient ERK time course appeared to substantially correspond to the behavioral response induced by morphine.

In looking at the time course of behavioral activity within the 30 min test sessions, the highest level of activity in the vehicle group is always during the first 5 min. In that this can be considered as reflective of the highest level of activation in brain dopamine systems, then this would appear be the time interval when morphine inhibition of GABA inhibitory neurons in the NAc and VTA would most strongly enhance the ongoing release of mesolimbic dopamine (Johnson and North, 1992; Vanderschuren et al., 1999; Lüscher and Ungless, 2006). In our time course study of morphine induced changes in ERK, we did find that morphine induced the largest ERK effects in these encephalic areas 5 min after the morphine injections.

When connecting the ERK increases in reward related brain areas during the first 5 min to the behavioral increase in activity in the first 5 min post-injection, we suggest that the behavioral increase in activity could be viewed as a reward-mediated effect. In linking a morphine reward effect to the increase in locomotor activity in the present study, it is the initial prepotent spontaneous activity which then becomes associated with the morphine reward effect. Numerous operant conditioning studies have demonstrated that when delivery of morphine in made contingent on a behavioral response, then the behavioral response rate is increased (Silva et al., 2007). In the present study there was no explicit response contingency associated with the delivery of the morphine injection. While this arrangement clearly does not fit into the conventional operant conditioning paradigm, nevertheless it can be incorporated into an expanded operant conditioning conceptualization. In the present study the initial exposure to the test arena reliably and immediately elicited locomotor activity so that the concurrent onset of morphine activation of the brain reward systems would lead to a reliable pairing of the drug reward effect with the behavioral response of locomotor activity. This association can be considered as analogous to the superstitious behavior described by Skinner (Reynolds, 1975), wherein the non-contingent delivery of food reward in pigeons resulted in the emergence of a reliable behavior pattern as a specific behavioral activity that was adventitiously associated with reward delivery. In the present study, following placement in the test arena, the initial pre-potent response was locomotor activity that would reliably occur in conjunction with the activation of brain reward systems by morphine. Therefore, as is generally the case, when responses are associated with reward, the response rates increase. In the present study the response is spontaneous activity rather than an experimenter selecting the response such as lever pressing that is typically employed in operant conditioning experiments.

If morphine-induced increases in locomotor activity with repeated treatments are categorized as an operantly conditioned behavioral response, then the increase in locomotor activity is an indirect and not a direct drug effect. That is, the direct drug effect is on the encephalic reward system, whereas the behavioral effect is on the ongoing non-drug behavior. In the present report, morphine was administered just prior to the animal being placed in the test arena where the prepotent response was locomotor activity, so this response became opportunistically associated with the onset of morphine activation of brain reward systems. Thus, in using increased activity in the VTA and NAc as proxies for morphine reward effects, then morphine induces locomotor stimulation simply because locomotor activity is the prepotent behavior in this paradigm. Presumably then if another behavior were prepotent then that behavior would increase. From this perspective, not only are the behavioral effects of the repeated morphine treatments indirect but, in addition, the activation of brain reward systems by morphine is also indirect by the disinhibition of dopamine neurons in brain reward systems.

Numerous operant conditioning studies have demonstrated that extinction can be substantially prolonged if the reward is administered intermittently. Thus, an implication of this alternative operant conditioning analysis of the progressive increase in locomotor activity with repeated morphine injections is that, if, in the present study, injections were continued but on an intermittent schedule such as a variable ratio schedule versus being administered before every test session then, when injections were discontinued, persistence of an increased activity response in non-drug tests would be considerably prolonged in the intermittent group despite receiving fewer morphine injections.

Although we did not find an increase in locomotor activity with the initial morphine injection in the first 30 min morphine test or in the 5 min test in the additional ERK experiment, we did find that in the fifth 30 min test session that the VEH and MOR-UP groups that received morphine did exhibit an increase in locomotor activity in the initial 5 min of the 30 min test session. While this observation appears in opposition to the absence of morphine induced increases in locomotor activity in the first 5 min of the first 30 min test and the 5 min additional ERK experiment, this differential may be attributed to the decreased baseline activity level resulting from the habituation effect induced by the five 30 min test sessions. That is, the activity level in the first 5 min of the 30 min test sessions decreased substantially from session 1 to session 5. In fact, the increase in activity in the first 5 min induced by morphine in the VEH and MOR-UP groups in session 5 only restored activity to their level of activity in the first 5 min of session 1. That is, before habituation. It has been long established (Järbe et al., 1981) that many psychoactive drugs have potent stimulus properties. Therefore, when the VEH and MOR-UP groups received morphine prior to test session 5, the habituation that had developed with respect to the test environment stimuli now, after MOR, the test environment stimuli also included the morphine drug stimuli. Seemingly, this introduction of new stimuli (i.e. drug stimuli) to the test environment disinhibited the established habituation and the activity level returned to pre-habituation level.

In conclusion, a consideration of the behavioral sensitization effects of repeated morphine injections in terms of operant conditioning not only offers a new perspective on this phenomenon but also has heuristic

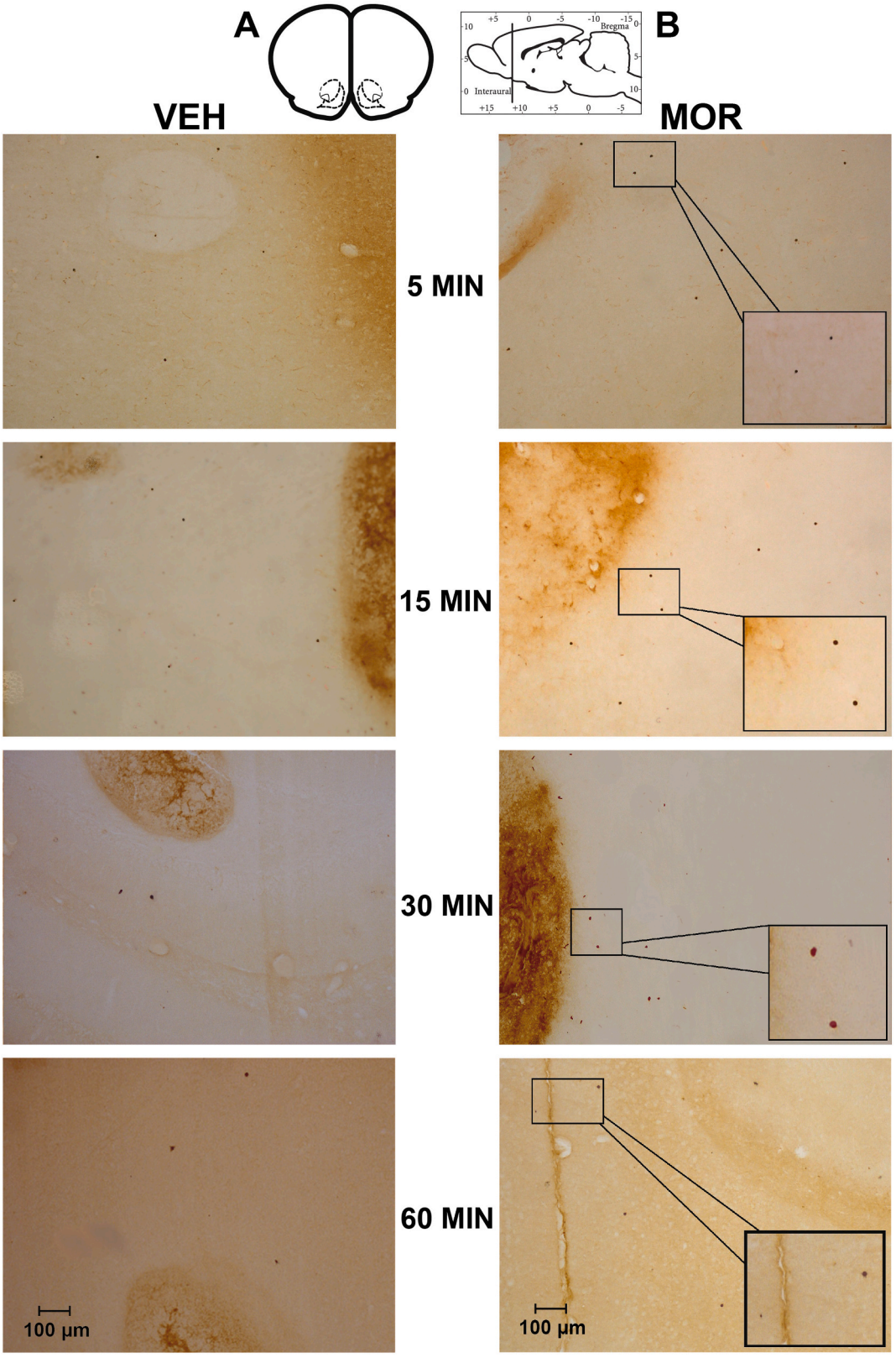

Fig. 5. Representative low (10×) and high (40× inserts) magnification images of ERK-P-immunoreactive cells from the NAc 5, 15, 30 and 60 min after VEH and MOR administration for the time course experiment. Drawings of Sagittal section (B) displaying, at the vertical black bar, the location of coronal section (A) were obtained from the Paxinos and Watson atlas (2005). Scale bar indicates 100 µm in the low magnification images.

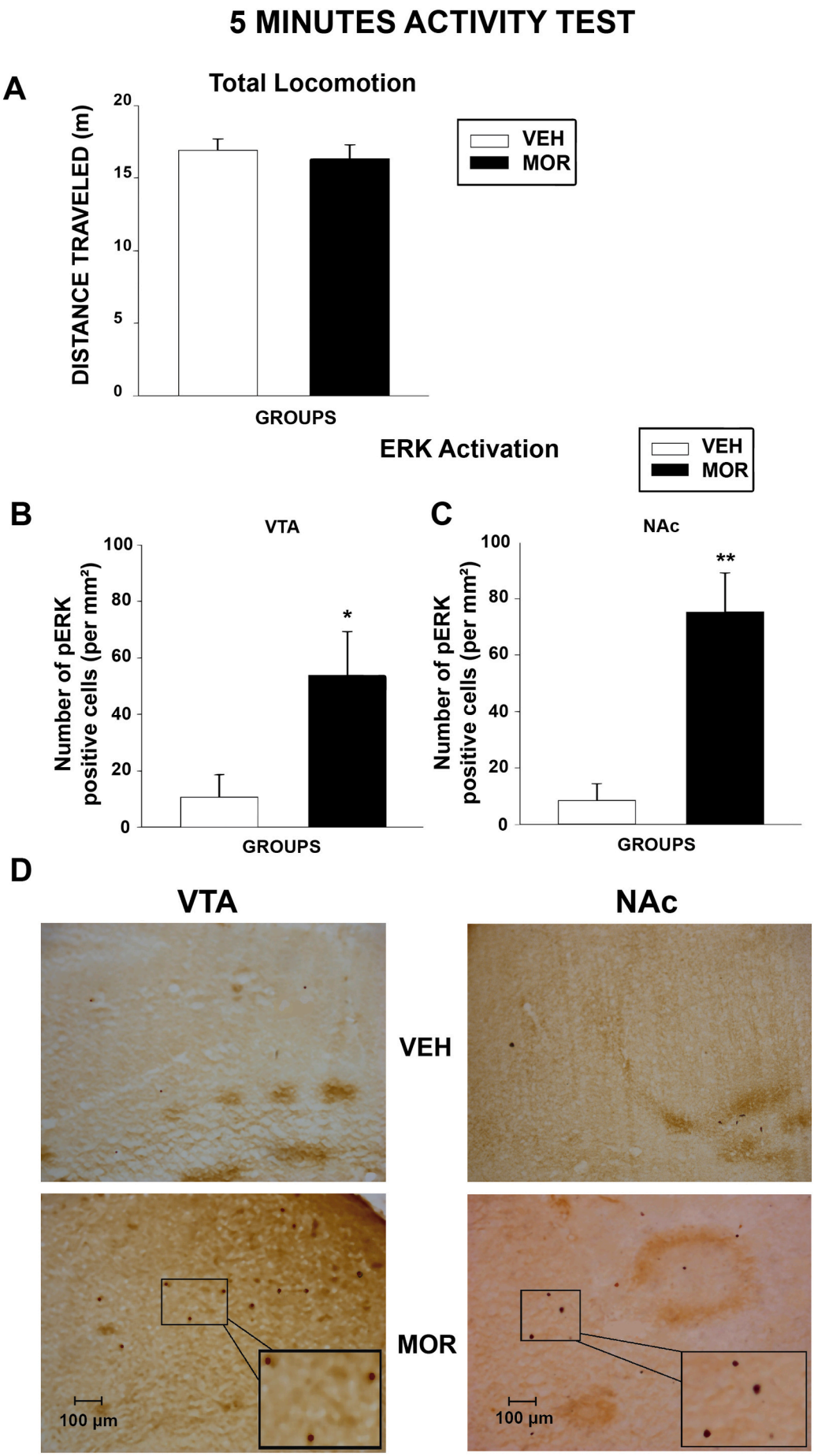

Fig. 6. Five minute activity test experiment. **A:** Means and S. E. M. of locomotor activity during pharmacological treatment with morphine 10 mg/kg and vehicle. **B** and **C:** Quantification of immunohistochemical results for ERK phosphorylation in the VTA (B) and NAc (C) following morphine 10.0 mg/kg and vehicle treatments. Data represents the mean ± S.E.M. ** ($p < 0.01$) and * ($p < 0.05$) denote higher numbers of immunoreactive nuclei in the MOR group when compared to the VEH group (independent t-test). **D:** Representative 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 low magnification images.

value in generating new lines of experimentation. In addition, morphine conditioned responses during abstinence are an important contributor to treatment resistance in the clinical management of opioid addiction. Therefore, elucidation of the possible role of operant conditioning processes associated with intermittent drug usage may be important for understanding the persistence of cue elicited drug responses that can trigger relapse during abstinence.

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References

Anagnostaras, S.G., Robinson, T.E., 1996. Sensitization to the psychomotor stimulant effects of amphetamine: modulation by associative learning. Behav. Neurosci. 110, 1397–1414. https://doi.org/10.1037//0735-7044.110.6.1397.

Bloise, E., Carey, R.J., Carrera, M.P., 2007. Behavioral sensitization produced by a single administration of apomorphine: implications for the role of pavlovian conditioning in the mediation of context-specific sensitization. Pharmacol. Biochem. Behav. 86, 449–457. https://doi.org/10.1016/j.pbb.2007.01.002.

Carey, R.J., Gui, J., 1998. Cocaine conditioning and cocaine sensitization: what is the relationship? Behav. Brain Res. 92, 67–76. https://doi.org/10.1016/s0166-4328(97)00126-5.

de Mello Bastos, J.M., Leite Junior, J.B., Samuels, R.I., Carey, R.J., Carrera, M.P., 2020. Post-trial low dose apomorphine prevents the development of morphine sensitization. Behav. Brain Res. 380, 112398 https://doi.org/10.1016/j.bbr.2019.112398-.

Di Chiara, G., Imperato, A., 1988. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc. Natl. Acad. Sci. U. S. A. 85, 5274–5278. https://doi.org/10.1073/pnas.85.14.5274.

Fricks-Gleason, A.N., Marshall, J.F., 2011. Role of dopamine D1 receptors in the activation of nucleus accumbens extracellular signal-regulated kinase (ERK) by cocaine-paired contextual cues. Neuropsychopharmacology 36, 434–444. https://doi.org/10.1038/npp.2010.174.

Järbe, T.U., Sterner, U., Hjerpe, C., 1981. Conditioning of an interoceptive drug stimulus to different exteroceptive contexts. Psychopharmacology 73, 23–26. https://doi.org/10.1007/BF00431094.

Johnson, S.W., North, R.A., 1992. Opioids excite dopamine neurons by hyperpolarization of local interneurons. J. Neurosci. 12, 483–488. https://doi.org/10.1523/JNEUROSCI.12-02-00483.1992.

Leite Junior, J.B., de Mello Bastos, J.M., Samuels, R.I., Carey, R.J., Carrera, M.P., 2019. Reversal of morphine conditioned behavior by an anti-dopaminergic post-trial drug treatment during re-consolidation. Behav. Brain Res. 2019 (359), 771–782. https://doi.org/10.1016/j.bbr.2018.08.009.

Liu, X.S., Hou, Y., Yan, T.L., Guo, Y.Y., Han, W., Guan, F.L., Chen, T., Li, T., 2014. Dopamine D3 receptor-regulated NR2B subunits of N-methyl-d-aspartate receptors in the nucleus accumbens involves in morphine-induced locomotor activity. CNS Neurosci. Ther. 20, 823–829. https://doi.org/10.1111/cns.12276.

Lu, L., Xu, N.J., Ge, X., Yue, W., Su, W.J., Pei, G., Ma, L., 2002. Reactivation of morphine conditioned place preference by drug priming: role of environmental cues and sensitization. Psychopharmacology 159, 125–132. https://doi.org/10.1007/s002130100885.

Lüscher, C., Ungless, M.A., 2006. The mechanistic classification of addictive drugs. PLoS Med. 3, e437 https://doi.org/10.1371/journal.pmed.0030437.

Marin, M.T., Berkow, A., Golden, S.A., Koya, E., Planeta, C.S., Hope, B.T., 2009. Context-specific modulation of cocaine-induced locomotor sensitization and ERK and CREB phosphorylation in the rat nucleus accumbens. Eur. J. Neurosci. 30, 1931–1940. https://doi.org/10.1111/j.1460-9568.2009.06982.x.

Matsui, A., Jarvie, B.C., Robinson, B.G., Hentges, S.T., Williams, J.T., 2014. Separate GABA afferents to dopamine neurons mediate acute action of opioids, development of tolerance, and expression of withdrawal. Neuron 82, 1346–1356. https://doi.org/10.1016/j.neuron.2014.04.030.

Matthews, R.T., German, D.C., 1984. Electrophysiological evidence for excitation of rat ventral tegmental area dopamine neurons by morphine. Neuroscience 11, 617–625. https://doi.org/10.1016/0306-4522(84)90048-4.

Mattingly, B.A., Gotsick, J.E., Salamanca, K., 1988. Latent sensitization to apomorphine following repeated low doses. Behav. Neurosci. 102, 553–558. https://doi.org/10.1037//0735-7044.102.4.553.

Nasello, A.G., Machado, C., Bastos, J.F., Felicio, L.F., 1998. Sudden darkness induces a high activity-low anxiety state in male and female rats. Physiol. Behav. 63, 451–454. https://doi.org/10.1016/s0031-9384(97)00462-9.

Neisewander, J.L., Bardo, M.T., 1987. Expression of morphine-conditioned hyperactivity is attenuated by naloxone and pimozide. Psychopharmacology 93, 314–319. https://doi.org/10.1007/BF00187249.

Paxinos, G., Watson, C., 2005. The Rat Brain in Stereotaxic Coordinates, 6th edition. Elsevier Academic Press, New York.

Post, R.M., Weiss, S.R., Fontana, D., Pert, A., 1992. Conditioned sensitization to the psychomotor stimulant cocaine. Ann. N. Y. Acad. Sci. 654, 386–399. https://doi.org/10.1111/j.1749-6632.1992.tb25983.x.

Powell, K.R., Holtzman, S.G., 2001. Parametric evaluation of the development of sensitization to the effects of morphine on locomotor activity. Drug Alcohol Depend. 62, 83–90. https://doi.org/10.1016/s0376-8716(00)00167-8.

Reynolds, G.S., 1975. A Primer of Operant Conditioning, Rev. ed. Scott, Foresman.

Sanguedo, F.V., Dias, C.V., Dias, F.R., Samuels, R.I., Carey, R.J., Carrera, M.P., 2016. Reciprocal activation/inactivation of ERK in the amygdala and frontal cortex is correlated with the degree of novelty of an open-field environment. Psychopharmacology 233, 841–850. https://doi.org/10.1007/s00213-015-4163-z.

Sanguedo, F.V., Dias, F.R., Bloise, E., Cespedes, I.C., Giraldi-Guimarães, A., Samuels, R.I., Carey, R.J., Carrera, M.P., 2014. Increase in medial frontal cortex ERK activation following the induction of apomorphine sensitization. Pharmacol. Biochem. Behav. 118, 60–68. https://doi.org/10.1016/j.pbb.2013.12.020.

Sanguedo, F.V., Figueiredo, A.M., Carey, R.J., Samuels, R.I., Carrera, M.P., 2017. ERK activation in the prefrontal cortex by acute apomorphine and apomorphine conditioned contextual stimuli. Pharmacol. Biochem. Behav. 159, 76–83. https://doi.org/10.1016/j.pbb.2017.07.011.

Sanguedo, F.V.C., Samuels, R.I., Carey, R.J., Carrera, M.P., 2019. Medial prefrontal cortex ERK and conditioning: evidence for the association of increased medial prefrontal cortex ERK with the presence/absence of apomorphine conditioned behavior using a unique post-trial conditioning/extinction protocol. Behav. Brain Res. 365, 56–65. https://doi.org/10.1016/j.bbr.2019.02.022.

Sharf, R., Guarnieri, D.J., Taylor, J.R., DiLeone, R.J., 2010. Orexin mediates morphine place preference, but not morphine-induced hyperactivity or sensitization. Brain Res. 1317, 24–32. https://doi.org/10.1016/j.brainres.2009.12.035.

Silva, M.T.A., Gonçalves, F.L., Garcia-Mijares, M., 2007. Neural events in the reinforcement contingency. Behav. Anal. 30, 17–30. https://doi.org/10.1007/BF03392140.

Vaher, K., Anier, K., Jürgenson, M., Harro, J., Kalda, A., 2020. Cocaine-induced changes in behaviour and DNA methylation in rats are influenced by inter-individual differences in spontaneous exploratory activity. J. Psychopharmacol. 34, 680–692. https://doi.org/10.1177/0269881120916137.

Valjent, E., Pascoli, V., Svenningsson, P., Paul, S., Enslen, H., Corvol, J.C., Stipanovich, A., Caboche, J., Lombroso, P.J., Nairn, A.C., Greengard, P., Hervé, D., Girault, J.A., 2005. Regulation of a protein phosphatase cascade allows convergent dopamine and glutamate signals to activate ERK in the striatum. Proc. Natl. Acad. Sci. U. S. A. 102, 491–496. https://doi.org/10.1073/pnas.0408305102.

Vanderschuren, L.J., Schoffelmeer, A.N., Mulder, A.H., De Vries, T.J., 1999. Dopaminergic mechanisms mediating the long-term expression of locomotor sensitization following pre-exposure to morphine or amphetamine. Psychopharmacology 143, 244–253. https://doi.org/10.1007/s002130050943.

Vezina, P., Stewart, J., 1984. Conditioning and place-specific sensitization of increases in activity induced by morphine in the VTA. Pharmacol. Biochem. Behav. 20, 925–934. https://doi.org/10.1016/0091-3057(84)90018-2.

Weisberg, S.P., Kaplan, G.B., 1999. Adenosine receptor antagonists inhibit the development of morphine sensitization in the C57BL/6 mouse. Neurosci. Lett. 264, 89–92. https://doi.org/10.1016/s0304-3940(99)00188-3.