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Morphine administered post-trial can induce potent conditioned morphine effects

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ABSTRACT

Morphine has substantial pro-dopamine effects and in rodents, this is expressed in behavior as increased locomotor activation. Here we administered post-trial 3 dose levels of morphine (3.0, 5.0 and 10.0 mg/kg) or vehicle either immediately or after a 15 min delay to different groups of rats following a brief (5 min) exposure to a novel test environment. Three post-trial injections were administered on three successive days. One day after the first post-trial morphine injections, the non-drug activity levels in the immediate post-trial morphine treatment groups were selectively increased compared to vehicle groups. The activity effects were potentiated with repeated immediate post-trial morphine treatments but the same morphine treatments given after a 15 min post-trial delay did not increase activity in any tests and did not differ from vehicle. Subsequently, all groups were given 5 daily non-drug test sessions as an extinction protocol. The increased activity levels in the 5.0 and 10.0 mg/kg immediate post-trial morphine groups were sustained over the five extinction sessions. Two days later all groups were given a 30 min non-drug test and the 5.0 and 10.0 immediate post-trial groups continued to exhibit a heightened level of activity relative to vehicle restricted to the initial 10 min of the test session. There were no other group differences. The findings that the locomotor stimulant effects in the immediate post-test morphine groups occurred on non-drug tests and that the same morphine treatments given 15 min post-test were without effect are consistent with a conditioned morphine effect. In that acquisition of familiarization with a new environment is a basic learning process that engages consolidation mechanisms, it is possible that the immediate post-trial morphine effects that occur concurrently with consolidation can become incorporated into this consolidation process and subsequently be expressed as a conditioned drug effect.

1. Introduction

The use of a contextual conditioned stimulus (CS) that is paired/unpaired to drug treatment acting as an unconditioned stimulus (UCS)/unconditioned response (UCR) has come to be a widely used drug conditioning paradigm to investigate stimulant drug induced changes in complex motor behaviors. Initially, Pickens and Dougherty (1971), Tilson and Rech (1973) and Schiff (1982) demonstrated that the locomotor stimulant effects of dopaminergic drugs such as amphetamine could be conditioned to test environment cues. This use of drug-induced changes in spontaneous motor behavior as a dependent behavioral variable has been employed in numerous studies to assess conditioned drug behavior (Anagnostaras and Robinson, 1996; Carey and Gui, 1998; Pickens and Dougherty, 1971; Post et al., 1992). We (Bloise et al., 2007; Braga et al., 2009a, 2009b; Damianopoulos and Carey, 1992; de Matos et al., 2010) along with others (Mattingly et al., 1997, 1988; Rowlett et al., 1991) have shown that in rats locomotor stimulant doses of apomorphine induce locomotor hyper-activity and that these effects undergo conditioning and sensitization. Recently, Santos et al. (2015, 2017, 2018) using a conventional post-trial consolidation protocol instead of the conventional pre-test drug conditioning protocol found that an immediate but not a delayed post-trial apomorphine treatment induced conditioned apomorphine effects. Specifically, it was shown that a low autoreceptor dose (0.05 mg/kg) of apomorphine that induced locomotor inhibition as well as high locomotor stimulant dose (2.0 mg/kg) of apomorphine administered shortly after a brief exposure to a novel

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environment induced conditioned hypo and hyper-activity, respectively. If the same post-trial injections were delayed 15 min post-trial then the treatments are equivalent to vehicle injections. These immediate post-test effects were only observed if the test environment was novel. If the same treatments were administered immediately post-trial after brief exposure to a familiar environment no conditioning occurred (de Mello Bastos et al., 2014; Leite Junior et al., 2018). In conventional drug conditioning wherein the drug effects are experienced in the testing environment, it is possible to argue for Pavlovian response conditioning but this is clearly not the case for post-trial drug conditioning in that the drug induced responses are not experienced in the test environment. The post-trial apomorphine findings, however, appear consistent with effects on consolidation processes. The seminal study by Duncan (1948) that showed that electroconvulsive shock (ECS) administered right after a learning trial for a new behavior prevented learning but if the post-trial ECS was delayed then the learning was unaffected. In addition, if the ECS was administered immediately after a test trial but after learning was already complete then the ECS was without effect since the memory was already consolidated. In that, consolidation occurs in the initial but not late stages of learning a new behavior (Duncan, 1948), it was assumed in the apomorphine post-trial studies that consolidation would occur following a novel environment experience in that there was no prior consolidated memory for the testing environment whereas, for the familiar environment consolidation had already occurred. Thus, the post-trial apomorphine seemingly could be incorporated into the active consolidation process following exposure to a novel environment but not following exposure to a familiar environment when the consolidation process was assumed to be relatively inactive.

In that these post-trial conditioned drug effects (Santos et al., 2018) were limited to apomorphine, the objective of the present study was to extend this investigation of post-trial drug conditioning to another psychoactive drug morphine. In mice and rats, morphine induces locomotor stimulation effects that undergo sensitization and conditioning with repeated treatments (Lu et al., 2002; Neisewander and Bardo, 1987; Powell and Holtzman, 2001; Sharf et al., 2010; Scheggi et al., 2000; Tzschentke and Schmidt, 1995; Vanderschuren et al., 1997, 1999; Vezina and Stewart, 1984). Recently, we have reported (Leite Junior et al., 2018) locomotor conditioning and sensitization effects in rats with repeated morphine (10.0 mg/kg) pre-test treatments. In the present study, we administered repeated morphine treatments post-test following a brief exposure to a novel environment in an immediate/delay post-test protocol to assess whether conditioned morphine locomotor stimulant effects were induced and whether they were selectively induced in the immediate post-test morphine groups.

2. Materials and 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 07:00 h and off at 19:00 h). All experiments occurred between 14:00 and 18:00 h. For 7 days prior to all experimental procedures, each animal was weighed and handled daily for 5 min. All experiments were conducted in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and in accordance with the Brazilian Society of Neuroscience and Behavior (SBNeC) for the care and use of laboratory animals.

2.2. Drugs

Morphine sulfate (Cristalia, SP, Brazil) was used from 10 mg ampoules (1 ml) and was injected subcutaneously in the nape of the neck at doses of 3, 5 and 10 mg/kg (Powell and Holtzman, 2001; Sharf et al., 2010; Vanderschuren et al., 1997; Vanderschuren et al., 1999). 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 experiments 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 travelled (m), was automatically analyzed using EthoVision software (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. Testing under red light conditions is less stressful and favors locomotor activation as the rats are transferred from the ambient light of the vivarium to the red light of the testing room (Nasello et al., 1998). 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 procedure

The experiments were conducted following a modified protocol from Santos et al. (2015). There were two experimental post-trial treatment conditions: immediate (I) versus 15 min delay (D) post-trial treatments. For both the immediate and delay post-trial groups, all rats received pre-trial vehicle injections immediately before being placed into the experimental arena for 5 min. For the immediate post-trial groups, injections were made straight after removal from the test environment following completion of the test session (I-POST). For the immediate post-trial treatments, the rats were equally subdivided into four groups in which one group received vehicle (VEH-I-POST $n = 7$), a second group received morphine 3 mg/kg (MOR-3-I-POST; $n = 7$), a third group received morphine 50 mg/kg (MOR-5-I-POST; n = 7) and a fourth group received morphine 10 mg/kg (MOR-10-I-POST; n = 7). The induction phase was conducted on three successive days. On the following day, the extinction phase was initiated, during which all animals only received vehicle injections pre and post-trial. There were 5 extinction sessions of 5 min duration with one session conducted per day. Three days later the final 30 min test was conducted. This 30 min test was conducted in order to determine if a generalized change in behavior occurred or whether the conditioned effects were limited to the initial exposure to the test environment. The delay post-trial treatments followed the same protocol, except that the post-trial injections were administered 15 min after removal from the test environment (D-POST). The four delayed groups were: VEH-D-POST ($n = 7$), MOR-3-D-POST (n = 7), MOR-5-D-POST (n = 7) and MOR-10-D-POST (n = 7). Table 1 and Table 2 present, respectively, the experimental timeline and the experimental groups.

2.5. Statistics

For the induction and extinction phase results, a two-way mixed design ANOVA with repeated measures was used to analyze the locomotor data to determine the group effect, day effect, as well as the interactions between variables. When a significant effect of group versus day interaction was recorded, data were further analyzed by one-way ANOVA followed by the Duncan post-hoc test and by paired t-test. For the behavioral data obtained from the final test, the total time of test was divided into 6 intervals of 5 min each and the data were

Table 1
Timeline.

DAYS	1	2	3	4	5	6	7	8	9	10	11
	Induction Phase			Extinction Phase					Withdrawal Period		Final Test

analyzed using a two-way ANOVA with repeated measures and when a significant interaction was found the results were analyzed using a one-way ANOVA. Wherever indicated by the ANOVA (group effects with *p*-values < 0.05), possible differences among groups were analyzed by Duncan's multiple range test.

3. Results

Fig. 1 shows the locomotor activity during the induction phase for the immediate and 15 min delay post-trial experiments. For the immediate post-trial experiment (Fig. 1A), a repeated two-way ANOVA showed an interaction group X day [F (6, 48) = 11.41; $p < 0.01$], an effect of groups [F (3, 24) = 10.65; $p < 0.01$] and an effect of days of treatment [F (2, 48) = 23.33; $p < 0.01$]. A one-way ANOVA followed by Duncan's multiple range test was used to further analyze the interaction of group X days. The one-way ANOVAS showed that on day 1, there was no differences among the groups [F (3, 24) = 0.80; $p > 0.05$]. From day 2 until day 3, the morphine groups had higher locomotion than vehicle group ($p < 0.05$). On day 2 [F (3, 24) = 11.4; $p < 0.01$], all morphine groups had higher locomotion distance scores than the vehicle group but there was no difference among the morphine groups ($p > 0.05$). However, on day 3 [F (3, 24) = 18.60; $p < 0.01$], the morphine 10 mg/kg group had higher locomotion than the morphine 3 mg/kg group ($p < 0.05$). There was no difference between the morphine 10 mg/kg and morphine 5 mg/kg groups ($p > 0.05$) and no difference between morphine 5 mg/kg and morphine 3 mg/kg groups ($p > 0.05$). For the comparison between the induction test days 1 and 3, the paired *t*-test showed that for the morphine 3 mg/kg [t (6) = 8.0; $p < 0.01$], morphine 5 mg/kg [t (6) = 5.91; $p < 0.01$] and morphine 10 mg/kg [t (6) = 5.0; $p < 0.01$] groups, the locomotion increased above the initial response level to the novel environment. For the 15 min delay post-trial experiment (Fig. 1B), a repeated two-way ANOVA showed that there was only an effect of days of treatment [F (2, 48) = 46.0; $p < 0.01$]. There was no effect of groups [F (3, 24) = 0.08; $p > 0.05$) and no interaction group X day [F (6, 48) = 1.70; $p > 0.05$]. It is also relevant to note that on day 1 there were no differences among the 8 groups [F (7, 48) = 0.76; $p > 0.05$].

Fig. 2 shows the locomotor activity during the extinction phase for the immediate and 15 min delay post-trial experiments. For the immediate post-trial experiment (Fig. 2A), a repeated two-way ANOVA showed only an effect of groups [F (3, 24) = 10.82; $p < 0.01$]. There was no effect of days of treatment [F (4, 96) = 0.92; $p > 0.05$] and no interaction groups X days [F (12, 96) = 0.31; $p > 0.05$]. The one-way ANOVA showed that the morphine groups had higher locomotion than the vehicle group ($p < 0.05$). However, the morphine 10 mg/kg group had higher locomotion than the morphine 5 mg/kg and morphine 3 mg/kg groups ($p < 0.05$) and the morphine 5 mg/kg group had higher locomotion than the morphine 3 mg/kg group ($p < 0.05$). For the 15 min delay post-trial groups (Fig. 2B), a repeated two-way ANOVA showed that there was no effect of groups [F (3, 24) = 1.82; $p > 0.05$], no effect of days of treatment [F (4, 96) = 0.50; $p > 0.05$] and no interaction groups X days [F (12, 96) = 0.43; $p > 0.05$].

Fig. 3 shows the locomotor activity of during the final test for the immediate and 15 min delay post-trial experiments. In order to evaluate the within session analysis, the locomotion score of the final test was divided into 6 intervals of 5 min. For the immediate post-trial treatment groups (Fig. 3A), a repeated two-way ANOVA showed an interaction of groups X intervals [F (15, 120) = 4.63; $p < 0.01$], an effect of intervals [F (5, 120) = 88.74; $p < 0.01$] but no effect of groups [F (3, 24) = 2.50; $p = 0.088$]. A one-way ANOVA followed by Duncan's multiple range test to further analyze the interaction of group X days showed that during interval 1, the morphine 10 mg/kg had higher locomotion than morphine 3 mg/kg and the vehicle groups ($p < 0.05$). There was no difference between the morphine 10 mg/kg and morphine 5 mg/kg groups ($p > 0.05$). The results also showed that the morphine 5 mg/kg had higher locomotion than the vehicle group ($p > 0.05$). There was no difference between the morphine 5 mg/kg and morphine 3 mg/kg groups ($p > 0.05$) and no difference between morphine 3 mg/kg and vehicle

Table 2
Experimental groups.

Initial groups	Induction phase: Immediate pre-arena	Induction phase: Post-arena	Extinction phase: Immediate pre-arena	Extinction phase: Post-arena	Final test: Immediate pre-arena	Final groups
Immediate post-trial treatments						
VEH	VEH	VEH	VEH	VEH	VEH	VEH-I-POST (n=7)
MOR-3	VEH	MOR-3	VEH	VEH	VEH	MOR-3-I-POST (n=7)
MOR-5	VEH	MOR-5	VEH	VEH	VEH	MOR-5-I-POST (n=7)
MOR-10	VEH	MOR-10	VEH	VEH	VEH	MOR-10-I-POST (n=7)
15 min. delay post-trial treatments						
VEH	VEH	VEH	VEH	VEH	VEH	VEH-D-POST (n=7)
MOR-3	VEH	MOR-3	VEH	VEH	VEH	MOR-3-D-POST (n=7)
MOR-5	VEH	MOR-5	VEH	VEH	VEH	MOR-5-D-POST (n=7)
MOR-10	VEH	MOR-10	VEH	VEH	VEH	MOR-10-D-POST (n=7)

VEH= vehicle; MOR-3= morphine 3 mg/kg; MOR-5= morphine 5 mg/kg; MOR-10= morphine 10 mg/kg; I= immediate; D=delay.

VEH = vehicle; MOR-3 = morphine 3 mg/kg; MOR-5 = morphine 5 mg/kg; MOR-10 = morphine 10 mg/kg; I = immediate; D = delay.

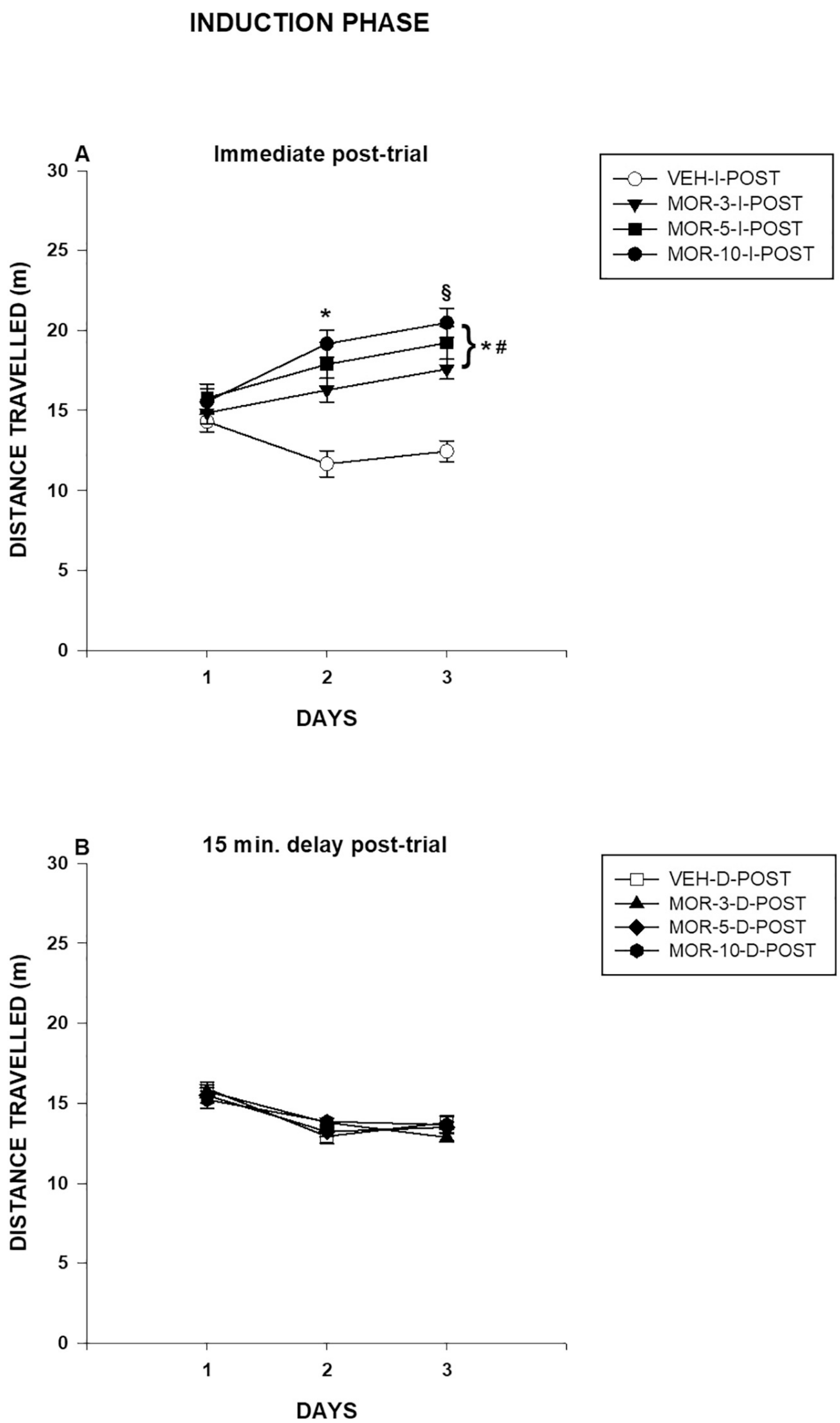

Fig. 1. Means and S. E. M.s for distance travelled (m) during in the 3-successive daily 5 min induction phase for the immediate (A) and 15 min delay groups (B). * $p < 0.05$ versus vehicle group (one-way ANOVA on each day followed by Duncan's multiple range test). § $p < 0.05$ morphine 10 mg/kg versus morphine 3 mg/kg group (one-way ANOVA followed by Duncan's multiple range test). # $p < 0.01$ first induction day versus the third induction day for each morphine groups (paired *t*-test).

group ($p > 0.05$). During interval 2, the morphine 10 mg/kg had higher locomotion than the other groups ($p < 0.05$), except for the morphine 5 mg/kg groups ($p < 0.05$). There was no difference between the morphine 5 mg/kg and morphine 3 mg/kg groups ($p > 0.05$) and no difference between morphine 3 mg/kg and vehicle group ($p > 0.05$). From interval 3 through interval 6, there were no differences among the groups ($p > 0.05$). For the 15 min delay post-trial (Fig. 3B), a repeated two-way ANOVA showed only an effect of intervals [$F_{(5, 120)} = 101.54$; $p < 0.01$]. There were no group effects [$F_{(3, 24)} = 0.60$; $p > 0.05$] and no interaction groups X intervals [$F_{(15, 120)} = 0.83$; $p > 0.05$].

4. Discussion

In the present study, it is important to recognize that on day 1 all groups were closely matched in terms of the locomotor activity levels and that the drug treatments were not initiated until after day1 test sessions were completed. Therefore, drug effects would only be manifested on day 2 following the post-test treatments that were administered after day 1. Thus, the finding that conditioned drug effects were evident on day 2 indicated that conditioning was induced after only one immediate post-trial treatment. Furthermore, the conditioned response

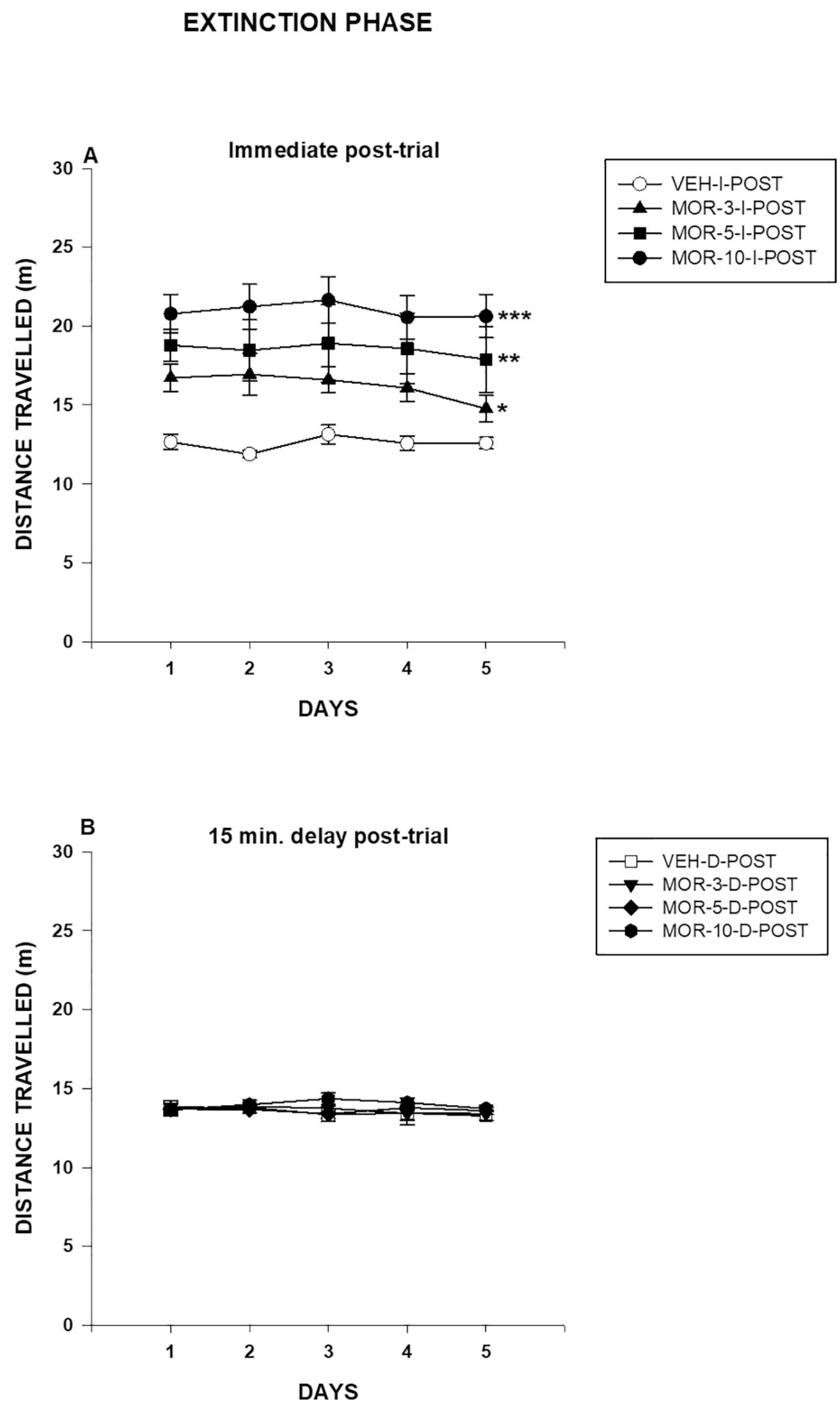

Fig. 2. Means and S. E. M.s for distance travelled (m) during the 5-successive daily 5 min extinction phase for immediate (A) and 15 min delay groups (B). *** $p < 0.05$ versus all groups. ** $p < 0.05$ versus 3.0 mg/kg morphine and vehicle group, * $p < 0.05$ versus vehicle group (one-way ANOVA followed by Duncan's multiple range test).

increased with repeated treatments whereas the morphine delay treatments were without effect and were equivalent to the vehicle treatment. It is also of importance that the immediate morphine post-trial treatments increased activity above the initial novel environment response level indicating that the effect was a positive conditioned effect rather than an interference effect on habituation. If the immediate post-trial morphine treatment effects simply increased activity levels above the vehicle group then it could be argued that the morphine treatments interfered with consolidation so that the environment remained novel. The final 30 min test showed that the conditioned effects were restricted to the initial temporal phase of the test environment placement. In that the post-trial treatments were associated with the initial placement, this outcome is consistent with a conditioned effect. Indeed, in the last 20 min of the test, the activity of the groups did not differ and all groups showed typical within session habituation. Thus, the only apparent effect of the immediate post-test treatments was a conditioned effect and that otherwise the treatments did not have a generalized impact on behavior.

The present findings point to the importance of the immediate post-trial consolidation interval in that the morphine effects were not experienced in direct association with the test environment cues and that repeated administration of the same morphine treatments after a

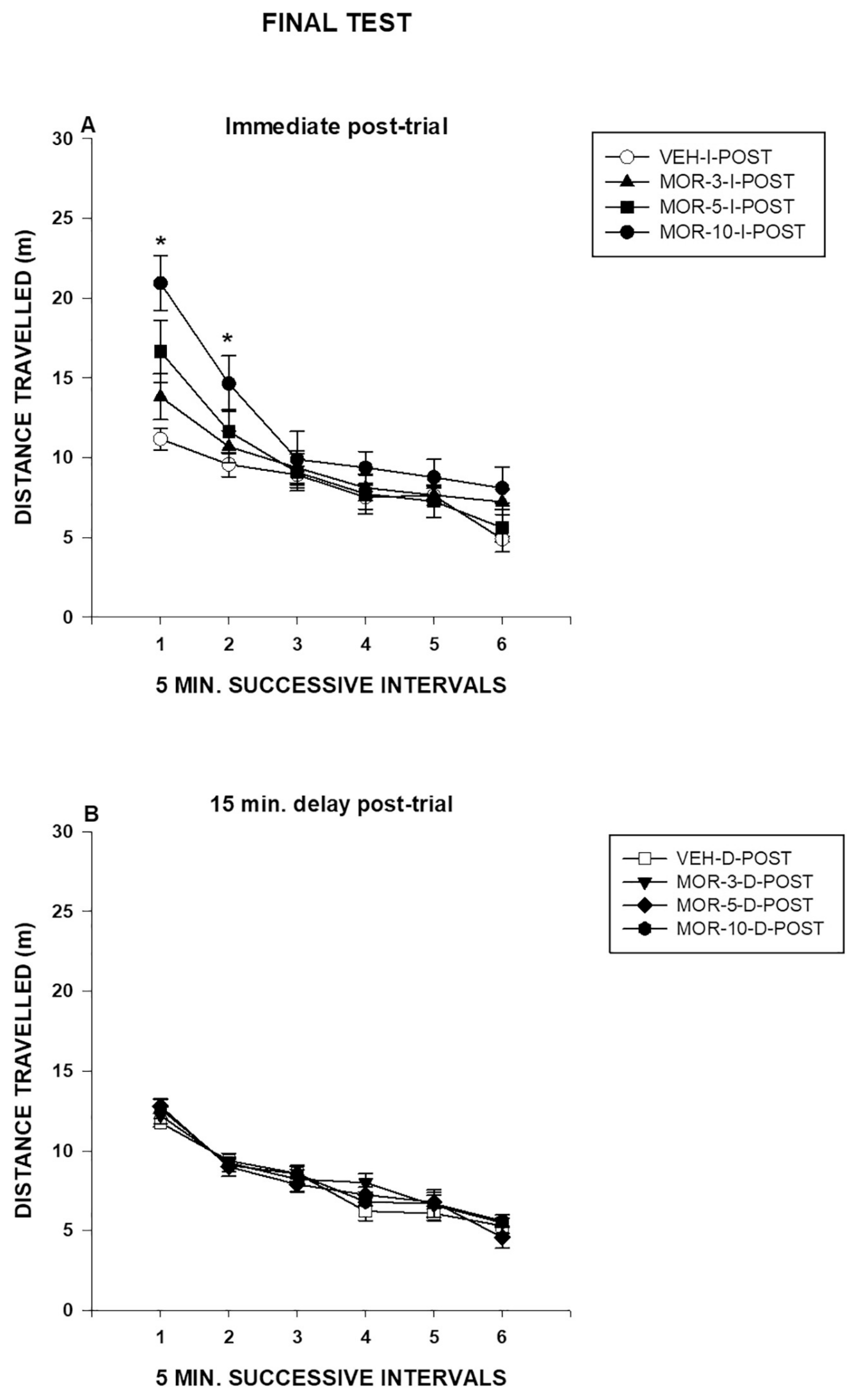

Fig. 3. Means and S. E. M.s for distance travelled (m) during in the 30 min final test for immediate (A) and 15 min delay groups (B). * $p < 0.05$ versus vehicle group (one-way ANOVA for each interval followed by Duncan's multiple range test).

15 min delay following removal from the test environment were without effect. While Duncan (1948) used ECS to demonstrate that there is a critical post-trial consolidation period, the severe and global impact of ECS on brain activity made such a manipulation less useful as a tool to uncover post-trial processes pertinent to memory that is necessary for retention. Insight into the various mechanisms that mediate post-trial treatment memory modulatory effects was developed from a large series of studies that showed that the post-trial period in the initial stages of acquiring a new behavior when the consolidation process was dynamic and could be modulated (Gasbarri and Tomaz, 2012; Gold et al., 1982; Messier, 2004; Roozendaal and McGaugh, 2011). A variety of post-trial systemic injections of treatments such as epinephrine, substance P and psychostimulants administered immediately post-trial following acquisition of a new behavior were shown to be able to substantially modify acquisition (Hasenöhrl et al., 1990; McGaugh and Roozendaal, 2009; Simon and Setlow, 2006; Wiig et al., 2009). The same treatments given post-trial after a short delay were without effect or if they were administered after the behavior had already been acquired. This sharp differential impact of immediate versus delayed post-trial treatment effects has provided the bedrock support for treatment modulation of consolidation. In that the delay treatments were closer in time to the subsequent non-drug learning trial than the immediate post-trial treatments, this absence of an effect of the same treatments given after a delay ruled out possible drug related non-associative factors including drug carry over to the next day non-treatment test. These studies have established a long-standing precedent for post-trial drug

treatments having a substantial impact on behavior. In these post-trial drug treatment investigations the objective was to modulate consolidation so that expression of the drug effects were indirect, namely enhancing/retarding of acquisition of an instrumental response. In contrast, in the post-trial apomorphine experiments (Santos et al., 2015, 2017, 2018) and the present experiment, the conditioned effects on behavior mirrored the direct effects of apomorphine and morphine respectively on locomotor behavior. That is, the conditioned behavioral effects on locomotor activity of pre/post-test apomorphine were the same (Santos et al., 2018). The findings are also in line with our recent report (Leite Junior et al., 2018) that morphine (10.0 mg/kg) administered immediately prior to a 30 min exposure to an open-field test arena led to a progressive increase in locomotion with repeated treatments and a conditioned hyper-locomotion response in subsequent 5 min conditioning tests. Furthermore, in the morphine treatment phase, the progressive increases in locomotion were evident in the initial 5 min of the test sessions and paralleled the increases observed for session totals. This latter finding indicates that the onset of the hyper-locomotion response elicited by morphine occurred within 5 min. In our previous studies with apomorphine, the onset of the locomotor stimulant effects occurred in < 2.5 min (de Mello Bastos et al., 2014). While the onset of the systemic apomorphine and morphine treatments is imprecise, evidently it is < 5 min. On the other hand, the temporal occurrence of consolidation in these types of behaviors is uncertain. In that the 15 min delay was sufficient to prevent conditioning, it is apparent that the consolidation interval was < 15 min. In order to provide a better estimate of the temporal parameters of the "black box" consolidation process in this behavioral model, a parametric study is needed in which the post-test intervals are systematically varied as an independent variable (eg. post-test intervals: immediate; 2 min; 4 min; 8 min).

While the most straightforward interpretation of the present finding from a consolidation perspective is that some indeterminate fraction of the morphine drug effect occurs during consolidation so that it is in effect incorporated as a component of the consolidated memory. Thus, on the subsequent exposure to the contextual cues, this morphine drug effect is evoked. In this respect, the present findings are in line with Pavlov's formulation of the conditioned response as a fractional replica of the unconditioned response (Pavlov, 1927).

An important relatively recent development in the understanding of conditioned drug effects has been the recognition that when a cue activated conditioned drug response is activated, the memory or association once again becomes labile, so that events that occur during reconsolidation can modify the association. While emphasis has been on ways to interfere with or attenuate the association during reconsolidation (Berman and Dudai, 2001; Debiec et al., 2002; Dudai, 2004; Eisenberg et al., 2003; Eisenhardt and Menzel, 2007; Leite Junior et al., 2018; Lee et al., 2006; Nader, 2003; Nader et al., 2000), it is also the case that the association can be strengthened by treatments that are administered during re-consolidation (Carey et al., 2014). Furthermore, we have shown that immediate but not delayed apomorphine treatments after the elicitation of an apomorphine conditioned response can potentiate/attenuate the conditioned apomorphine conditioned response (Carrera et al., 2011, 2012, 2013). In line with the latter consideration, the progressive increase in the conditioned response we observed with repeated post-trial morphine treatments could be seen as progressively strengthening the conditioning by the post-trial treatment effects occurring during re-consolidation. On the other hand, repeated exposures to the novel environment followed by vehicle injections would be expected to increasingly diminish the impact of the immediate post-test morphine treatments as a function of environment exposures as the environment becomes increasingly less novel. This consideration is amenable to experimental analysis by making a comparison of the effects of immediate post-test morphine treatments after (eg.1, 2, 4 or 8 exposures) to the novel environment. An implication of the possible progressive strengthening of the conditioned response by drug use during reconsolidation is that with repeated use of an addictive drug, a consolidation/reconsolidation cycle can occur such that the conditioned cues becomes increasingly strengthened and can acquire increasing control over behavior as the drug effects keep being reconsolidated with continued drug taking.

In conclusion, the present findings suggest that the association of morphine effects with the consolidation process can lead to the development of robust morphine conditioned effects. This suggests that drug taking in a new context can become associated with consolidation or drug taking in conjunction with cue evoked conditioned drug effects may become associated with re-consolidation. In both of these ways drug taking could potentially strengthen conditioned drug effects.

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