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Research report

Dose- and time-dependent, context-induced elevation of dopamine and its metabolites in the nucleus accumbens of morphine-induced CPP rats

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ABSTRACT

The dopamine (DA) projections from the ventral tegmental area to the nucleus accumbens (NAc) are the key component of the brain reward circuitry. The encoded information by DA in reward-related memory within this circuit during opiate reinforcement requires further clarification. The present study was designed to explore the correlations between morphine dose, retention of morphine-induced conditioned place preference (CPP), morphine-induced changes in levels of DA and its metabolites in the NAc in expression and retention of CPP in Sprague–Dawley male rats. A dose-effect curve for morphine-induced CPP (0.01–10 mg/kg, i.p.) was obtained using 4-day conditioning sessions followed by a CPP test; the retention of morphine CPP was measured with CPP tests after the development of CPP. We found a dose-dependent effect of morphine (from 0.01 to 10.0 mg/kg, i.p.) on both the magnitude and the retention of CPP. During the retention of morphine-induced CPP, a morphine-dose- and time-dependent elevation of DA and its metabolites was observed in the NAc. These changes were absent if the same dose of morphine was injected outside of the conditioning environment (i.e., in the home cage). These results suggest that that the long-lasting elevation of DA and its metabolites in the NAc is attributable mainly to drug-associated context, rather than the residual effect of morphine.

1. Introduction

Ingestion of drugs of abuse has long-lasting behavioral consequences, one of which is that drug-related behaviors can be elicited and maintained by stimuli associated with the effects of the drug. In the absence of the drug itself, these conditioned stimuli (CSs) have been demonstrated to maintain and renew drug-seeking behavior in rats [1–3]. Drug-associated CSs maintain their effectiveness long after the period of acute withdrawal from drugs in rats [4] and in humans [5]. Most of the drugs abused by humans produce conditioned place preference (CPP) in animals. Thus, CPP has become a widely used animal model for the study of the rewarding properties of abused substances such as opiates and cocaine [6].

The mesolimbic dopaminergic system (MLDS) mediates pathological behavioral changes that occur with repeated exposure to drugs of abuse [7,8]. In the MLDS, dopaminergic neurons originating in the ventral tegmental area (VTA) project to the nucleus accumbens (NAc), a key neural substrate for reinforcement of drug-seeking behavior underlying addiction to morphine [9,8]. Opiates elevate dopamine levels in the NAc [10], and the elevated dopamine leads to neural adaptation that underlies reinforcement and addiction to morphine in animals and humans [7,9].

What information is encoded by dopamine activity in the MLDS of subjects addicted to drugs? Debate continues on the precise contributions made by MLDS to rewards and reward-associated context [11]. An early view of DA function was that it signaled experience of addictive drugs as a hedonic neurotransmitter, but this viewpoint has been challenged by pharmacological blockade, genetic, and lesion studies [12,13] in which animals preserved preference for rewards such as sucrose even after dopamine depletion. All behavioral tests in the present study were performed in drug-free status, which is helpful to exclude the plausible explanation of changed dopamine activity made by drugs of abuse. Instead of acting as a hedonic signal itself, we hypothesize that increased dopamine activity is a conditioned response to reward-related context.

The present experiments were designed (1) to investigate the relationship between the dose of morphine used in CPP conditioning, the magnitude of CPP expression and the duration of CPP retention, (2) to examine changes in NAc content of DA and its metabolites dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA) in CPP rats, and (3) to assess the effects of alternate

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morphine/saline injections, without the CPP conditioning, on the content of DA and its metabolites in the NAc.

2. Materials and methods

2.1. Animals

All experiments were performed on male Sprague–Dawley rats, weighing 180–220 g at the beginning of the experiment, obtained from Experimental Animal Center, Peking University. Animals were housed 4 per cage in a 12:12 h light/dark cycle (lights on at 07:00) with food and water available at all times. The room temperature was maintained at 22 ± 1 °C. Animals were conditioned and tested during the light phase of the cycle. They were handled daily during the first week after arrival. All experimental procedures were approved by the Animal Use Committee of the Peking University Health Science Center and in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Apparatus

Conditioning was conducted in black rectangular polyvinyl chloride boxes (795 mm × 230 mm × 250 mm), containing three chambers separated by guillotine doors [14–16]. The two large black conditioning chambers (A and C, 280 mm × 220 mm × 225 mm) were separated by a small gray center choice chamber B (135 mm × 220 mm × 225 mm). Chamber A has 4 light-emitting diodes (LEDs) forming a square on the wall and a stainless steel mesh floor (22.5 mm × 22.5 mm), chamber C has 4 LEDs forming a triangle on the wall and a stainless-steel rod floor (15 mm apart), whereas chamber B has a gray wooden floor. Fourteen photobeams 47.5 mm apart were placed across the chambers. Through a computer interface, the time spent in each chamber was recorded for each rat.

2.3. Place preference procedure

A total of 80 rats were used to test the dose effect of morphine on CPP expression (see Table 1). The methods of CPP have been described in detail previously [14–16]. Briefly, animals received a single preconditioning test in which they were placed in the center choice chamber with the guillotine doors removed to allow access to the entire apparatus for 15 min. The amount of time spent in each chamber was monitored and used to assess natural preferences. The next day, rats were assigned to receive saline or morphine (0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10 mg/kg) paired with one of the two conditioning environments in a counterbalanced manner (the 'unbiased' procedure). All animals were confined to the lateral chambers for a period of 45-min twice daily with an interval of 6 h (09:00 and 15:00) for 4 days. The morphine groups received morphine injection in the morning session and saline injection in the afternoon session; whereas the saline groups received saline injections in both sessions. Morphine-paired sides were counterbalanced among all groups. The center choice chamber was never used during conditioning and was blocked by guillotine doors. After the 4-day conditioning, rats were placed in the center choice chamber with access to the entire apparatus for 15 min. The locomotor activity was estimated by counting the total number of crossings between any two adjacent compartments.

2.4. Retention of developed CPP

A total of 146 rats were used to investigate the temporal course of the retention of CPP induced by morphine at two dosages, 0.3 and 3.0 mg/kg (see details in Table 1). The rats were divided into 2 batches. The first batch of 67 rats was randomly divided into 6 groups, including 3 control groups conditioned by saline and 3 CPP groups conditioned by 0.3 mg/kg morphine. On the 2nd, 4th and 8th days after the last injection of conditioning drug, CPP tests were performed.

The second batch of 79 rats was randomly divided into 7 groups, plus one more saline group from the first batch, including 4 control groups conditioned by saline and 4 CPP groups conditioned by 3 mg/kg morphine. On the 8th, 16th, 32nd and 64th day after the last injection of conditioning drug, CPP tests were performed.

Table 1
Experimental design.

	Morphine (mg/kg)	Conditioning	Sampling[a]	*n*	Group no.
Experiment 1					
CPP induced by morphine in a dose-dependent manner	0	Yes	1	12	1
	0.01	Yes	1	10	2
	0.03	Yes	1	10	3
	0.1	Yes	1	10	4
	0.3	Yes	1	10	5
	1	Yes	1	9	6
	3	Yes	1	9	7
	10	Yes	1	10	8
Experiment 2					
CPP retention by low dose of morphine	0	Yes	1	12	As #1
	0.3	Yes	1	10	As #3
	0	Yes	2	10	9
	0.3	Yes	2	11	10
	0	Yes	4	12	11
	0.3	Yes	4	12	12
	0	Yes	8	12	13
	0.3	Yes	8	10	14
CPP retention by high dose of morphine	0	Yes	1	12	As #1
	3	Yes	1	9	As #7
	0	Yes	8	12	As #13
	3	Yes	8	10	15
	0	Yes	16	11	16
	3	Yes	16	11	17
	0	Yes	32	12	18
	3	Yes	32	11	19
	0	Yes	64	12	20
	3	Yes	64	12	21
Home-cage injection	0	No	1	6	22
	3	No	1	6	23
	0	No	8	6	24
	3	No	8	6	25
	0	No	16	6	26
	3	No	16	6	27
	0	No	32	6	28
	3	No	32	6	29

[a]For the conditioned groups, sampling shows how many days after the last conditioning, CPP test and brain dissection were performed; for groups by the "home-cage injections", shows how many days after the last injection tissue was harvested (see Section 2 for more details about "home-cage injections").

2.5. Tissue dissection and preparation

Rats were returned to their home-cage immediately after the CPP test and decapitated 1 h after the test. The brains were removed and placed on an ice-cooled plate for dissection of the NAc according to the stereotaxic atlas of Paxinos and Watson [17]. Immediately after, the tissue samples were weighed and placed in 1.5 ml plastic tubes containing ice-cold perchloric acid (200 μl, 0.4 M), homogenized for 10 s using ultrasound (0.5 Hz) and centrifuged for 20 min at 15,000 × *g* at 4 °C. The supernatant was passed through a 0.2 μm filter and kept at 4 °C until HPLC analysis.

2.6. Home-cage drug administration

A total of 48 rats were injected at home-cage following the same schedule of drug administration as in CPP groups, but without any behavior components of CPP training and testing. Four groups ($n = 6$ in each group) received daily injections of morphine (3 mg/kg) at 9:00 and saline at 15:00 for 4 days, and the other four groups ($n = 6$ in each group) received twice saline injections at 9:00 and 15:00, respectively. Tissue dissections were performed at the same time as that in CPP groups on the 1st, 8th, 16th, and 32nd days, respectively, after last injection.

2.7. HPLC analysis for dopamine and its metabolites

DA and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were analyzed using reversed-phase ion-pair chromatography combined with electrochemical detection under isocratic conditions [18]. The six-channel detector potentials were set at +50, 100, 200, 300, 400, and 500 mV using a glassy carbon electrode and an Ag/AgCl reference electrode. The mobile phase (0.6 mM 1-octanesulfonic acid, 0.27 mM Na_2EDTA, 0.043 M triethylamine and 50 ml acetonitrile/l, adjusted to pH 2.95 with H_3PO_4) was delivered at a flow rate of 0.5 ml/min onto the reversed phase column (125 mm × 3 mm with pre-column 5 mm × 3 mm, filled with Nucleosil 120-3 C18, Knauer, Berlin, Germany). Ten microliters aliquots were injected by an autoinjector with cooling module set at 4 °C. Data were calculated by an external standard calibration.

2.8. Statistical analysis

CPP score represents the index of place preference for each rat, calculated by dividing the time spent in the drug-paired compartment by the time spent in both conditioning compartments [19,15,16]. Concentrations of catecholamines in the NAc are presented as ng/mg tissue. Data were processed by commercially available software Graph Pad Prism 4.0. Results are presented as mean ± S.E.M. and analyzed with one-way ANOVA followed by Dunnett post-test (Figs. 1 and 2), two-way ANOVA followed by Bonferroni post-test (Figs. 3–5), and linear regression (Fig. 6). The accepted level of statistical significance is $P < 0.05$.

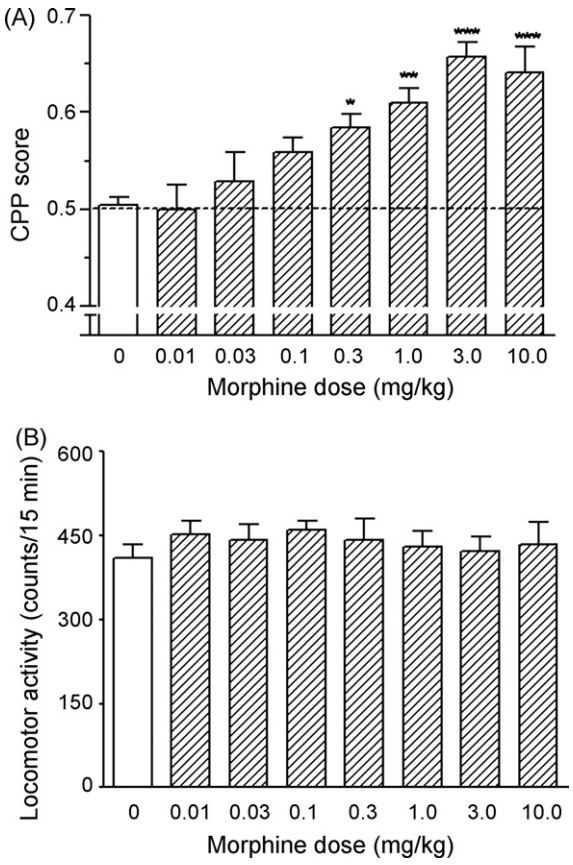

Fig. 1. Effect of morphine dose on the expression of CPP. (A) Different doses of morphine (0.01, 0.03, 0.1, 0.3, 1.0, 3.0 and 10 mg/kg) or saline (0 mg/kg morphine) were administered intraperitoneally in a 4-day schedule of conditioning. On the testing day, the animals were observed for a 15-min period. The CPP score for each rat was calculated by dividing the duration spent in the drug-paired compartment by the duration spent in both conditioning compartments. (B) The locomotor activity was assessed as described in Section 2. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, different from the saline (0 mg/kg morphine) control group, $n = 12$, 10, 10, 10, 10, 9, 9, and 10, respectively. For additional details, see Section 3.

3. Results

3.1. Experiment 1: CPP induced by morphine in a dose-dependent manner and DA/metabolites in NAc

3.1.1. Effect of the dose of morphine on the magnitude of CPP

The pre-conditioning test showed that animals spent almost an equal amount of time in the two end chambers (A: 311 ± 7.19 s, C: 314 ± 6.72 s) and less time in the small center choice chamber (B: 274 ± 10.84 s). There were no significant differences in the time spent in the two end chambers ($P > 0.05$). Thus, the CPP apparatus was considered as unbiased in terms of chamber preferences of untreated rats.

80 rats were used to explore the magnitude of morphine-induced CPP. Different doses of morphine (0, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0 and 10 mg/kg) were administered by intraperitoneal (i.p.) injection in the four sessions of conditioning. The CPP test was performed the following day. The relationship between the dose of morphine and the score of CPP is shown in Fig. 1A. One-way ANOVA revealed a significant dose-related preference [$F(7, 72) = 9.169$, $P < 0.0001$]. Significant place preference was observed in the groups treated with morphine at doses from 0.3 to 10 mg/kg, with maximum response obtained at 3.0 mg/kg of morphine. The doses of morphine used in the present study seemed not to affect the locomotor activity in the testing phase [$F(7, 72) = 0.3328$, $P = 0.9365$] (Fig. 1B).

3.1.2. Effect of morphine dose on the contents of DA and its metabolites in the NAc

As shown in Fig. 2, changes in DA content in the NAc as a function of dose of morphine strikingly paralleled morphine dose-dependent effects on expression of CPP (Fig. 1). One-way ANOVA analysis showed significant changes in the contents of DA [$F(7, 46) = 52.96$, $P < 0.0001$] as well as in DOPAC [$F(7, 44) = 9.698$, $P < 0.0001$] and HVA [$F(7, 44) = 10.12$, $P < 0.0001$]. A significant increase in the DA content appeared at the dose of 0.3 mg/kg morphine (by Dunnett post-test, $P < 0.05$), and reached a plateau from 1.0 to 10 mg/kg. The contents of DOPAC and HVA in the NAc were also significantly increased starting at the dose of 0.3 mg/kg morphine (by Dunnett post-test, $P < 0.05$), reaching a plateau from 0.3 to 10 mg/kg.

3.2. Experiment 2: retention of morphine-induced CPP and DA/metabolites in NAc

3.2.1. The retention of CPP over time

Fig. 3A shows the temporal course of retention of CPP induced by 0.3 mg/kg morphine. Two-way ANOVA indicated significant effects of the conditioning drug (morphine/saline) [$F(1, 81) = 12.6$, $P = 0.0006$] and the time [$F(3, 81) = 4.64$, $P = 0.0048$], with no significant effect of the interaction between the conditioning drug and

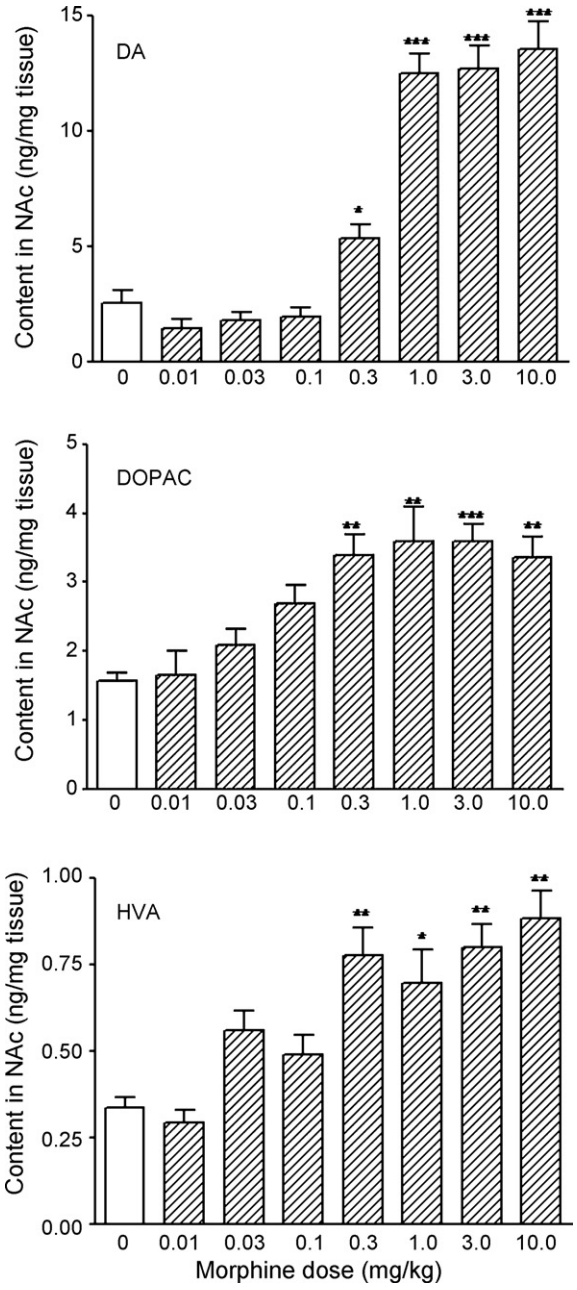

Fig. 2. Effect of morphine dose on DA and its metabolite levels in the NAc from CPP rats. The data were analyzed using one-way ANOVA followed by Dunnett post-test, $n = 8, 6, 6, 6, 8, 6, 8, 6$, respectively, in each group. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the saline (0 mg/kg morphine) control group.

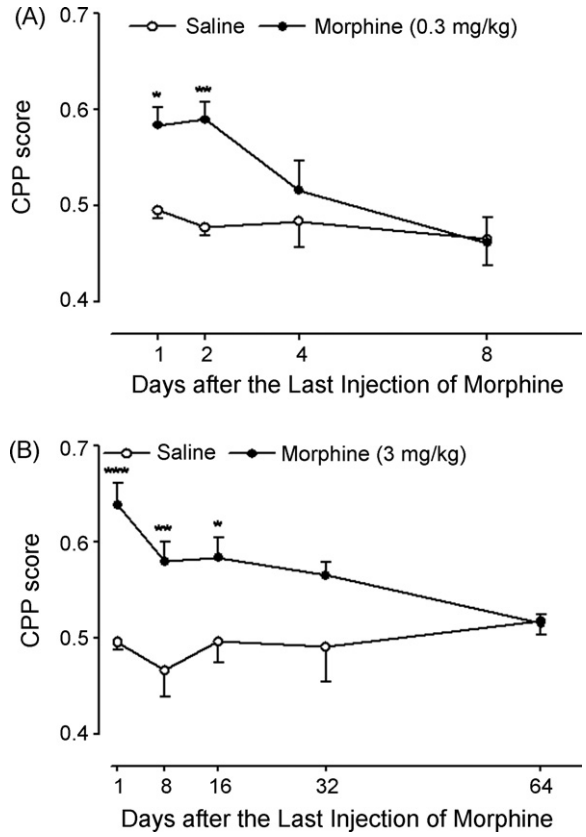

Fig. 3. Retention of morphine-induced CPP. All animals used in the experiments described in this section experienced only one test for place preference after CPP training. (A) Treated by 0.3 mg/kg morphine, $n = 10, 11, 12$ and 10, respectively. (B) Treated by 3.0 mg/kg morphine, $n = 11, 10, 12, 11$and 12, respectively. Values are expressed as mean ± S.E.M. of CPP score, calculated by dividing the time spent in the drug-paired compartment by the time spent in both conditioning compartments. The data were analyzed using two-way ANOVA followed by Bonferroni test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the corresponding saline group.

the time [$F(3, 81) = 2.61$, $P = 0.0568$]. Bonferroni post-test showed that the CPP remained for only 2 days, and disappeared on the 4th day.

Fig. 3B shows the curve for retention of CPP induced by 3 mg/kg morphine. Two-way ANOVA indicated significant effects of the conditioning drug (morphine/saline) [$F(1, 104) = 37.56$, $P < 0.0001$] and the interaction between the conditioning drug (morphine/saline) and the time [$F(4, 104) = 3.32$, $P = 0.0133$], although no significant effect of the time [$F(4, 104) = 1.75$, $P = 0.1456$]. Bonferroni post-test showed that the CPP induced by 3 mg/kg morphine was maintained for at least 16 days, with complete forgetfulness occurring 64 days after the last injection.

3.2.2. *Contents of DA and its metabolites in the NAc during the retention of CPP expression*

The tissue contents of DA and its metabolites in the NAc were tested on the CPP rats conditioned by morphine at 0.3 mg/kg. Two-way ANOVA analysis showed significantly increased tissue contents of DA [treatment $F(1, 56) = 13.93$, $P = 0.0004$; time $F(3, 56) = 3.26$, $P = 0.0282$; interaction $F(3, 56) = 4.40$, $P = 0.0076$] as well as its metabolites DOPAC [treatment $F(1, 48) = 47.25$, $P < 0.0001$; time $F(3, 48) = 10.26$, $P < 0.0001$; interaction $F(3, 48) = 7.26$, $P = 0.0006$] and HVA [treatment $F(1, 48) = 46.55$, $P < 0.0001$; time $F(3, 48) = 9.74$, $P < 0.0001$; interaction $F(3, 48) = 7.26$, $P = 0.0004$] in the NAc during the retention of the CPP. The contents of DA and its metabolites in the NAc peaked 2 days after the last injection of the drug, and returned to the baseline levels as represented by the saline control group 4–8 days after the last injection of morphine (Fig. 4).

The tissue contents of DA and its metabolites in the NAc were also tested on the CPP rats conditioned by morphine at 3.0 mg/kg. Two-way ANOVA showed significantly increased tissue contents of DA [treatment $F(1, 70) = 153.17$, $P < 0.0001$; time $F(4, 70) = 10.59$, $P < 0.0001$; interaction $F(4, 70) = 11.84$, $P < 0.0001$] as well as its metabolites DOPAC [treatment $F(1, 60) = 81.99$, $P < 0.0001$; time $F(4, 60) = 7.68$, $P < 0.0001$; interaction $F(4, 60) = 4.66$, $P = 0.0024$] and HVA [treatment $F(1, 60) = 50.53$, $P < 0.0001$; time $F(4, 60) = 23.48$, $P < 0.0001$; interaction $F(4, 60) = 4.80$, $P = 0.0020$] in the NAc during the retention of CPP (left panel of Fig. 5). Corresponding to the data shown in Fig. 3B, the baseline levels of DA, DOPAC and HVA in the control group shown in the left panel of Fig. 5 were simi-

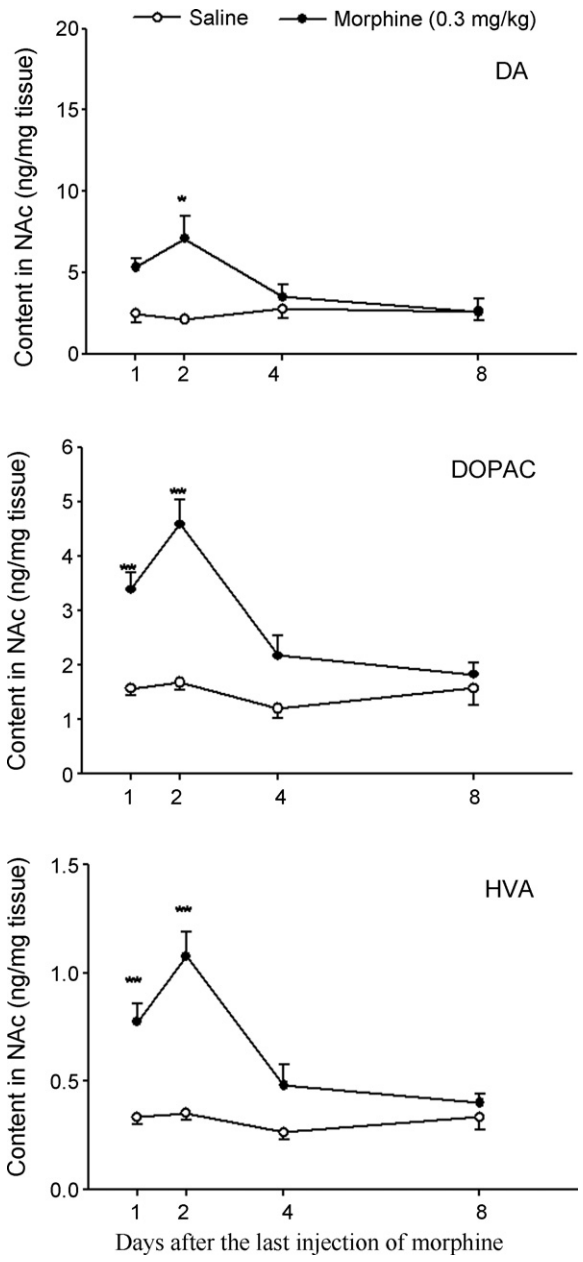

Fig. 4. Time-dependent change in tissue contents of DA and its metabolites in the NAc from CPP rats induced with 0.3 mg/kg morphine. The data were analyzed using two-way ANOVA followed by Bonferroni post-test, $n = 8$ in each group. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the corresponding saline group.

lar. In the morphine-induced CPP rats, the increase of DA content was much more drastic (4-fold increase in 3.0 mg/kg group and 2-fold increase in 0.3 mg/kg group), and longer lasting (32 days in 3.0 mg/kg group and 2 days in 0.3 mg/kg group). In contrast, the magnitude of change of the contents of DOPAC and HVA was quite similar (2–3-fold) in both groups, except that the changes last up to 16 days in the 3 mg/kg group, compared to 2 days in the 0.3 mg/kg group.

The right panel in Fig. 5 shows the contents of DA and its metabolites in rats injected alternately with 3 mg/kg morphine/saline (morphine group), or injected with saline twice a day (saline group), for four consecutive days, without CPP training or testing. Two-way ANOVA showed the contents of DA [treatment $F(1, 40) = 3.61$, $P = 0.0647$; time $F(3, 40) = 1.61$, $P = 0.2121$; interaction $F(3, 40) = 2.25$, $P = 0.0976$] as well as DOPAC [treatment $F(1, 40) = 1.26$, $P = 0.2689$; time $F(3, 40) = 3.31$, $P = 0.0296$; interaction $F(3, 40) = 5.92$, $P = 0.0019$] in NAc were not significantly increased by morphine treatment, although the change of HVA [treatment $F(1, 40) = 3.97$, $P = 0.0531$; time $F(3, 40) = 3.93$, $P = 0.0151$; interaction $F(3, 40) = 1.65$, $P = 0.1941$] 1 day after the last injection of morphine was significantly lower than that in saline group ($P < 0.01$, Bonferroni post-test after two-way ANOVA).

4. Discussion

Our data indicate that morphine produced a dose-dependent increase in place preference, in agreement with the literature [20], with higher doses of morphine used in the CPP training eliciting a larger magnitude of response to the drug-paired context in doses ranging from 0.01 to 10.0 mg/kg. However, since previous reports [20] showed that 1 mg/kg of morphine administered via the systemic route was required to induce significant place preference, we were surprised to find in our study that 0.3 mg/kg morphine was sufficient to produce significant place preference. In this context, it is noteworthy that the specially designed visual cues (LEDs forming different shapes) in combination with the tactile cues (different constructs of the floor) used in the CPP apparatus of the present study could facilitate discrimination between the two compartments.

A number of studies have explored the duration of retention of morphine-induced CPP [21,22]. In the present study, we investigated the retention of CPP induced by two different doses of morphine. The CPP induced by 0.3 mg/kg morphine lasted for at least 2 days, and disappeared completely 8 days after the last CPP conditioning, while that induced by 3 mg/kg lasted for at least 16 days, with a complete disappearance after 64 days. From these results we conclude that (1) while the fluctuation of CPP scores should not be excluded during behavioral test interval, CPP induced by a higher dose of morphine was significantly longer than that induced by a lower dose; and (2) the established morphine CPP is subject to forgetting after 1–9 weeks. The latter conclusion has been supported by the results obtained by Wang et al. [23] in our laboratory, showing that in the absence of extinction testing or extinction training, the morphine-induced CPP (4 mg/kg, i.p.) was no longer evident 7 days after the last conditioning trial.

In order to assess the importance of the MLDS in the mediation of the rewarding effect of morphine, we measured the contents of DA and its metabolites DOPAC and HVA in the NAc, and tried to correlate the neurochemical findings with the dynamic changes of the behavior, including the expression and retention of CPP in a dose- and time-dependent manner. The S-shaped tendency depicted in Fig. 2 almost coincides with those of behavioral changes shown in Fig. 1, with a turning point at 0.3 mg/kg and a plateau at 3–10 mg/kg. The 2–3-fold increase of the metabolites of DA (DOPAC and HVA) suggested an increased release or turnover of DA transmission. If the synthesis remained constant, then the content of DA should be decreased. In fact, there was a 7-fold increase of the DA content, suggesting that the synthesis was dramatically accelerated.

Having explored the dose response relationship, we proceeded to assess the time response relationship using two fixed doses of 0.3 and 3.0 mg/kg as the least and the maximally effective doses. Careful comparison of the data in the two figures revealed that the DOPAC- and HVA levels were essentially the same in the two dose settings, suggesting that the degree of release or catabolism of DA has been increased to a similar extent. In contrast, the content of DA in the NAc showed a marked difference, increasing 2-fold in the 0.3 mg/kg group and 4-fold in the 3 mg/kg group, suggesting that the rate of synthesis or anabolism was much higher in the high dose group. Even more noticeable was that the duration of the increase

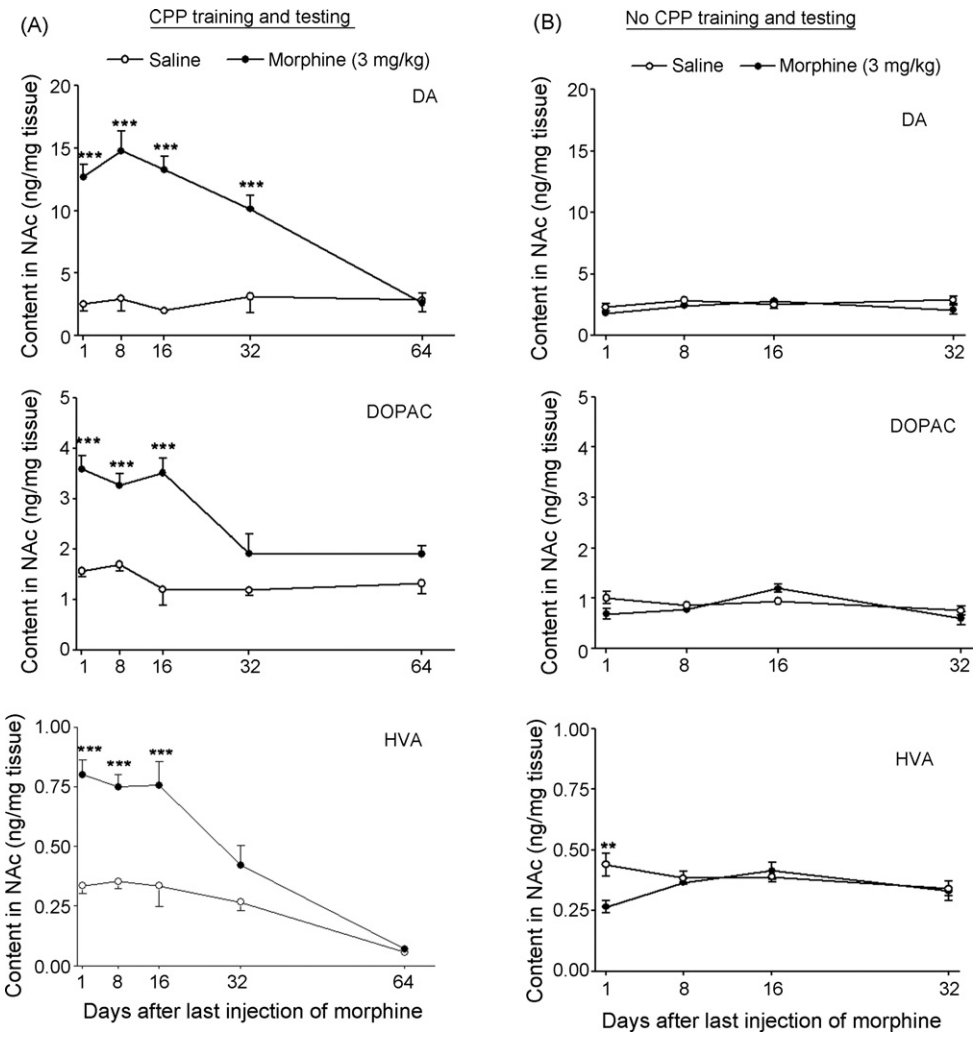

Fig. 5. Time-dependent change in tissue contents of DA and its metabolites in the NAc of rats injected with 3 mg/kg morphine. The data were analyzed using two-way ANOVA followed by Bonferroni post-test, $n = 8$ (A) or 6 (B) in each group. The left panel shows the transmitter contents in rats with CPP training and testing. The right panel shows the transmitter contents in rats only injected by morphine, without CPP training or testing. Data are expressed as mean ± S.E.M. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the corresponding saline group.

of the contents of DA and its metabolites showed a very high correlation with the CPP expression. In the 0.3 mg/kg group, the content peaked on the second day and returned to normal levels on the 4–8 days; whereas in the 3 mg/kg group the contents remained at a high level until the 16th day and faded away completely on the 64th day. It is difficult to assume that only 4 daily injections of such a small dose of morphine (0.3 and 3.0 mg/kg) would induce such a robust change in the synthesis and release of NAc DA with the effects outlasting the drug injection for 2 days and 2 weeks, respectively.

What then is the dominant factor determining the long-lasting neurochemical changes in the NAc? Two factors became obvious. One is the pharmacokinetics of morphine, and the other is the environmental conditioning. Experiments were designed to observe the effect of morphine on DA metabolism in the NAc. Rats were given daily alternate injections of morphine (3 mg/kg) and saline for 4 days, in much the same way as that in the CPP paradigm. The only difference was that the injections were made in the animal room with the rats returned to their home cage right after the injection. The assessment of DA metabolism in the NAc (Fig. 5, right panel) showed that there was no significant increase in the content of DA and its metabolites in the period of observation on days 1, 8, 16, 32 after the end of injection, except for a moderate decrease (instead of increase) of the HVA content on day 1. These results clearly indicate that the long-lasting up-regulation of DA activity could only be attributable to the consequence of conditioning rather than the residual effect of the drug. Concerning the decreased HVA in the NAc 24 h after last morphine injection, decreased DA turnover could be a reasonable explanation. The rats were handled and injected with morphine at the same time each day, thus counter-adaptive changes in DA might have already been acquired. The question here is why this only occurs in the HVA measure. To answer this question more studies are needed.

Considering the nature of the experiments performed in the present study, we cannot exclude alternative explanations for the increased tissue contents of DA and its metabolites. The increase in DA synthesis and release in the NAc, although displaying a strong correlation with CPP, may be attributable to some other conditioned effects of morphine such as conditioned locomotor sensitization, analgesia, gut motility or taste aversion. Furthermore, no place conditioning was seen at a morphine dose of 0.03 mg/kg in Experiment 1 of CPP expression and 32 days in Experiment 2 of CPP retention, but the tissue contents of HVA and DA in the NAc, respectively, were still higher than in control groups. Thus, the measure of DA and its metabolites in the NAc is more sensitive than the behavioral assessment of CPP score, which also suggests some other conditioned effects might be mediated by activation of MLDS. However, tissue

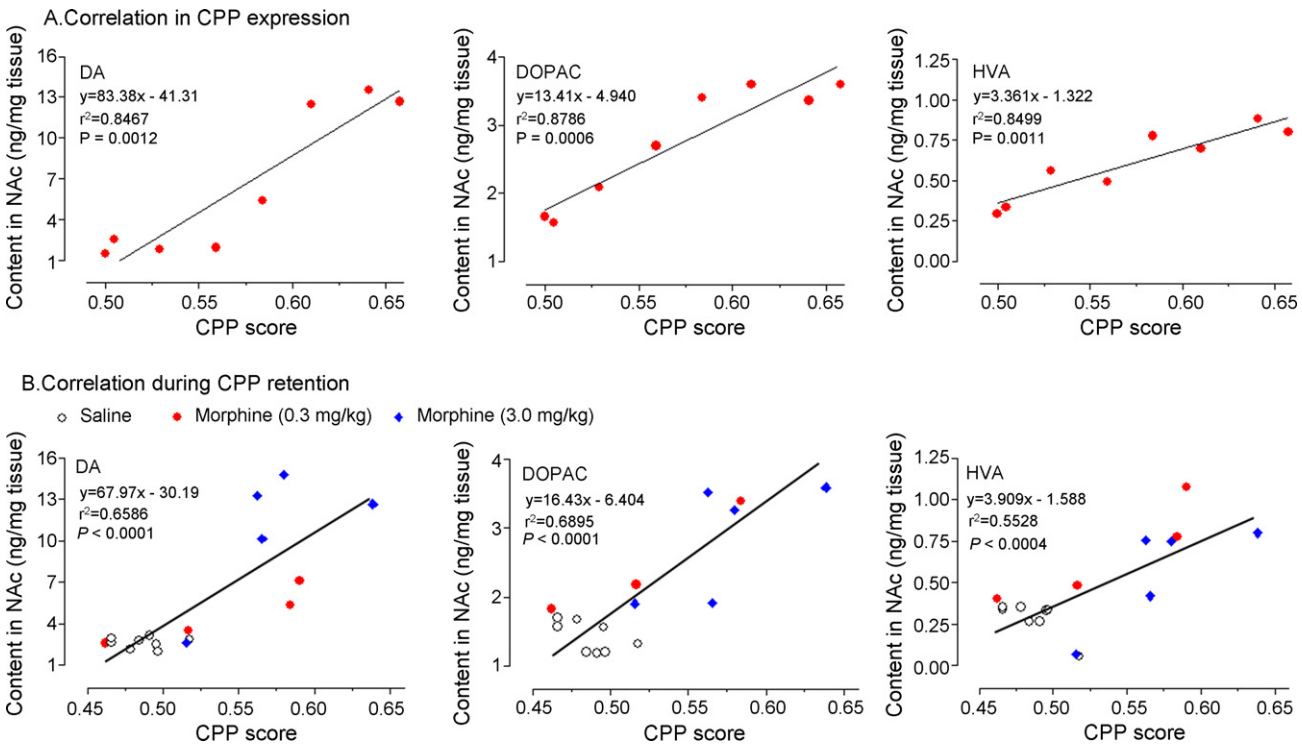

Fig. 6. Association between the tissue contents of DA and its metabolites and CPP scores. The location of each dot represents the mean value of tissue content and behavior score in a group conditioned by morphine at different doses (A) or tested on different days after conditioning; r^2 values refer to Pearson correlation coefficients; The trend lines are shown by functions as $y = ax + b$.

contents of DA and its metabolites presented strong correlations with CPP score in both CPP development ($r^2 > 0.84$ at least, $P < 0.01$ at least, Fig. 6A) and CPP retention ($r^2 > 0.55$ at least, $P < 0.001$ at least, Fig. 6B), which demonstrates a tight relationship between MDLS and CPP behavior.

Studies in our laboratory also revealed that rats conditioned with morphine/saline alternate injections and tested the following day, displayed no change in DA content in the NAc at 0 (i.e. no CPP test), 5 and 10 min after the beginning of CPP test, and a significant increase at 15 min after the beginning of CPP test (i.e. the end of CPP test) (data to be published). Taken together these results suggested that (1) CPP conditioning resulted in an inducible, rather than spontaneous, increase of the metabolism of DA in the NAc; (2) CPP rats were in a "readiness" status, rapidly responding to drug-associated context (within 15 min); (3) the response of CPP rats to context would be finally extinguished after a long period (2 months later).

It should be mentioned that the brain samples for neurochemical measurement were obtained 1 h after the end of CPP testing, or 75 min after the beginning of the exposure to reward-associated context. While it is easy to understand that the environmental cue would stimulate the release of DA from the nerve terminals, it is hard to reconcile how the synthesis of DA, which relied on the increased production of the rate limiting enzyme tyrosine hydroxylase (TH), can be accelerated within such a short time. Studies have indicated that several *trans*-acting factors (Fos, Jun, CREB, etc.) bind to *cis* elements within the promoter region of the TH gene and affect its transcription [24,25]. Cleavage of the proenzyme from the inactive form to functional form might be one possibility. In addition, the activity of this enzyme can be postulated to be up-regulated when morphine-induced CPP rats are exposed to the drug-associated context. Further studies are necessary to explore this issue.

In conclusion, our results clearly demonstrated that the dose of morphine bears a positive correlation with both the magnitude and the retention of response to drug-associated context. The increase of DA and its metabolites DOPAC and HVA in the NAc of CPP rats occurred in a morphine-dose- and time-dependent manner. The long-lasting influence on MLDS response was attributed mainly to drug-associated context, rather than the residual effect of morphine. A connection between drug-associated context, NAc DA and behavioral changes is postulated.

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