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Research article

Prenatal morphine exposure during late embryonic stage enhances the rewarding effects of morphine and induces the loss of membrane-bound protein kinase C-α in intermediate medial mesopallium in the chick

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HIGHLIGHTS

- Prenatal morphine during late stage of embryonic development may result in high risk of morphine rewards in progeny.
- The loss of membrane-bound protein kinase C-α of intermediate medial mesopallium is associated with increased susceptibility to morphine rewards in chicks prenatally exposed to morphine.

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ABSTRACT

The susceptibility to drug abuse may be associated with the structural and/or functional changes in the reward-related brain regions induced by drug exposure during sensitive periods of embryonic development. Previously, we have found that prenatal morphine exposure during embryonic days 17–20 may be crucial for developing the susceptibility to morphine reward after hatching. However, the underlying structure and cellular mechanisms need further investigation. In the present study, the chicks of a few days old, which were prenatally exposed to morphine during E17-20, obviously showed higher preference for the morphine-paired chamber and hyperactivity during the expression of morphine conditioned place preference (CPP), and the reduction in membrane-bound of PKCα of the bilateral intermediate medial mesopallium (IMM) assayed immunologically. These results indicate that the decreased expression of PKCα in IMM may participate in the development of the susceptibility to the rewarding effects of morphine in chicks prenatally exposed to morphine, and provide further support for the cross-species evolutionary concordance among amniotes.

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1. Introduction

There is growing evidence that prenatal exposure to addictive substances may induce the disturbances of embryonic development and then lead to neurobehavioral dysfunctions during postnatal life, including the alterations in addictive susceptibility [7,8,22,23,40,42]. Many studies on prenatal opiates exposure have indicated that the offspring may become [5,10,21,38,43,47] or fail to become [39,44] sensitized to opiates or other addictive agents in later life. Our previous experiments suggested that the late stage of embryonic development (E17-20) was the sensitive time-window when prenatal morphine exposure enhanced the susceptibility to morphine reward after hatch [13]. However, the cellular and molecular mechanisms underlying the complex process have not been fully clear yet.

The protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine protein kinases that play critical roles in the regulations of cell growth, differentiation, proliferation and signal transduction [17,34,37]. At least 10 distinct PKC isoforms can be classified into three subcategories: conventional PKCs (cPKC, α,

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βI, βII, γ), novel PKCs (nPKC, δ, ε, η, θ) and atypical PKCs (aPKC, ξ, I/λ) [35]. More recently, PKC has also been shown to play an important role in opiate addiction [11,31]. For example, the PKCγ translocation inhibitor γV5-3 and/or deletion of PKCγ gene could prevent the development of morphine-induced place preference in rodents [30,36]. Newton and colleagues have demonstrated that mice lacking PKCε are supersensitive to the rewarding effects of morphine, which are measured by means of conditioned place preference (CPP) and intravenous self-administration paradigms at very low doses of morphine [33]. Similar to PKCε knockout mice, Rodd and colleagues have revealed the decreased level of PKCα in the nucleus accumbens of rats, which have been trained to self-administration of ethanol [41]. Also, it has been found a reduction in the expression of PKCα in brain of heroin addicts and morphine-dependent rats [3,12]. These studies indicate that μ-opiate addiction may be related with the altered expressions of PKC isozymes in the brain reward system.

In the avian forebrain, the intermediate medial mesopallium (IMM) is crucial area for associative learning, where PKC and it's phosphorylated substrates have been widely investigated [2,48]. Moreover, it is well-known that drug addiction and learning and memory may be likely to share much in common in synaptic plasticity and related cellular and molecular mechanisms [18,19]. Our previous findings demonstrated that the acquisition of morphine-induced CPP was significantly blocked with the administration of 3 μg SCH23390 into the bilateral IMM before conditioning [14]. All these findings may suggest that PKC in the IMM of chick forebrain may also be implicated in the rewarding effects of morphine. Although all PKC isozymes except for PKCθ are expressed to varying degrees in the brain, the activities of the conventional α-isoform are the greatest in the central nervous system [20]. In the present study, using the procedure of CPP, we investigated whether prenatal morphine during E17-20 could alter the level of PKCα in the bilateral IMM in the young chicks, which were sensitized to morphine reward after hatch.

2. Materials and methods

2.1. Injection of morphine into eggs

Freshly fertilized "BAU-3" eggs (60 ± 5 g) were incubated in a domestic self-turning incubator (ShanDong BD Model 264 Incubator) with exposure to 12 h light/dark cycles. The incubator conditions were maintained at 37.8 °C and 55–65% relative humidity. 120 eggs were numbered and randomly assigned to two groups (n = 60/group): (1) embryonic eggs injected with morphine (1 mg/kg of egg weight, dissolved in 0.9% sterile saline, 20 μl/egg); (2) embryonic eggs injected with equivalent volumes of saline. Drug injections were once given in E17 and E19, as described by our previous findings [13]. Before drug injections the holes were drilled in the chorioallantois end of the eggshells. The microinjector needle was inserted to a depth of 0.5 cm. The holes were sealed with wax after injections.

2.2. Apparatus

The CPP test was conducted in four identical black Perspex chambers (70 × 30 × 30 cm), with two equal size end compartments (30 × 30 × 30 cm) separated by a black central area (10 × 30 × 30 cm). The 10 × 10 cm opening was centred at a lower part of the board between the central area and end compartment. The two end compartments had distinct floor colors: one green, the other red. The location and movement of each chick were automatically monitored and analyzed by the computer system. All experiments were carried out between 8 a.m. and 4 p.m. in the light phase of the cycle.

2.3. Conditioned place preference (CPP) paradigm

The subjects were assigned to three groups: (1) prenatally saline-exposed chicks were injected intraperitoneally with 0.9% physiological saline (5 ml/kg, n = 14), that were treated as a control group; (2) prenatally saline-exposed chicks were injected intraperitoneally with morphine (1 mg/kg, n = 14); (3) prenatally morphine-exposed chicks were injected intraperitoneally with morphine (1 mg/kg, n = 16). The CPP procedure consisted of a 6-day schedule with three phases: pretest, conditioning and posttest, as described by our previous studies [13,14]. Briefly, on postnatal day 1, all the chicks were placed individually into the central area and allowed to explore the apparatus freely for 900 s 9 chicks that stayed in the any compartment for more than 700 s or for less than 200 s were excluded from the remainder of the experiment. The subsequent conditioning phase lasted 45 min for 4 successive days, which were counterbalanced for a total of two saline pairings and two morphine pairings. Control group received saline injections on both sides. The CPP test started 24 h after the last conditioning training. The time spent and the distance travelled in each compartment were recorded during the posttest phase, which lasted 900 s. The laboratory protocol and procedure were carried out in accordance with the requirements and regulations of the Animal Care and Research Committee of Tianjin Medical University.

2.4. Immunoblotting of the PKCα isoform

The brains of chicks were removed immediately for dissection of the bilateral IMM samples after posttest. To obtain the tissue comprising much of IMM, the coronal cuts were placed approximately 1 mm lateral to the midline and this incision directed ventromedially towards the interhemispheric fissure. For more detailed information, the readers can refer to the studies by Watson [46]. The extracts of cytomembrane and cytoplasm fractions were prepared and estimated using the proteins extraction kits (Keygen biotechnology, Nanjing, China), and BCA assay respectively (Pierce, Rockford, IL). Equivalent amounts (50 μg) were transferred to nitrocellulose membranes (Millipore, USA) after electrophoresis. The membranes were blocked for 2 h at room temperature with 5% skim milk in Tris-buffered saline, pH 7.5, containing 0.1% Tween 20 (TBST). Subsequently, the membranes were incubated overnight at 4 °C with primary antibodies (P-PKCα, ab32502, 1:20000, Abcam, Cambridge, MA, USA), After washed thoroughly, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L) (BA1054, Boster Biotechnology, Wuhan, China) diluted 1:50000 in TBST containing 5% skim milk for 2 h at 37 °C. The antigen-antibody peroxidase complex was finally detected using an Enhanced Chemiluminescent kit (Thermo Fisher Scientific, Massachusetts, USA), and visualized by exposure to Kodak film (Eastman Kodak, Kodak, NJ). Integrated density values of PKCα were analyzed (Fluor Chen 2.0 software, Olympus, Yokohama, Japan) and normalized by the optical density of the corresponding GAPDH band. The values are presented as a ratio of the membrane or cytosol to GAPDH.

2.5. Statistical analysis

The time spent in the morphine-paired chamber of the apparatus during, before and after conditioning was analyzed by a two-way ANOVA for repeated measures on one factor. The within-subject factor was "test" ("pretest" versus "posttest"), and the between-subject factor was "prenatal treatment" ("saline" versus "morphine"). In case of significant interaction, analyses of simple

Table 1
Effects of prenatal morphine exposure on general characteristics of chicks.

Group	Hatch day	Hatch weight (g)	Hatch rate (%)	Sample numbers
Saline	20.89 ± 0.03	41.08 ± 0.24	84	50
Morphine	21.48 ± 0.05*	41.53 ± 0.20	75	45

Hatch day of the morphine group is longer than that of the saline group (*, P < 0.01). Chicks were weighed when being dried completely. Data are expressed as mean ± S.E.M.

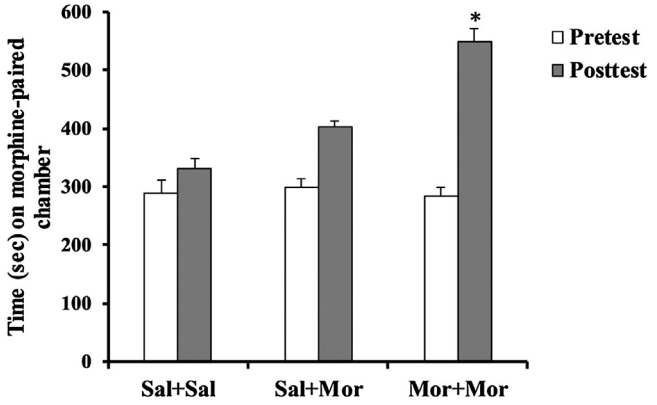

Fig. 1. The effects of prenatal morphine during E17-20 on the acquisition of morphine-induced place preference in chicks. Results are mean ± S.E.M. *, P < 0.01 versus "Sal + Sal" or "Sal + Mor" group. "Sal + Mor" group: prenatally saline-exposed chicks were injected intraperitoneally with saline; "Sal + Mor" group: prenatally saline-exposed chicks were injected intraperitoneally with morphine; "Mor + Mor" group: prenatally morphine-exposed chicks were injected intraperitoneally with morphine. N = 14-16 per treatment group.

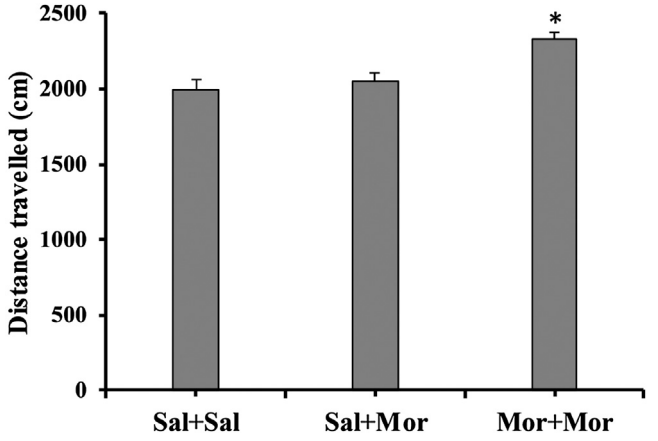

Fig. 2. The effects of prenatal morphine during E17-20 on locomotor activity in chicks during the CPP test. Locomotor activities were tested 24 h after the last conditioning phase. Results are mean ± S.E.M. *, P < 0.01 versus "Sal + Sal" or "Sal + Mor" group. "Sal + Sal" group: prenatally saline-exposed chicks were injected intraperitoneally with saline; "Sal + Mor" group: prenatally saline-exposed chicks were injected intraperitoneally with morphine; "Mor + Mor" group: prenatally morphine-exposed chicks were injected intraperitoneally with morphine. N = 14–16 per treatment group.

effects and post hoc tests (LSD) were performed to test differences between groups. The distance travelled in the apparatus during the posttest and the levels of PKCα after the posttest were compared among the groups by means of one-way analyses of variance (ANOVA) followed by post-hoc tests (LSD). Results from Table 1 and the difference in the basal expressions of PKCα of the IMM were determined by unpaired Student's *t*-test. Data are presented as mean ± S.E.M. The level of statistical significance was set at P < 0.05.

3. Results

3.1. The effect of morphine during E17-20 on the rewarding effects of morphine in chicks

There was a significant "prenatal treatment × test" interaction [F (2, 41) = 30.984, P < 0.01] among groups (Fig. 1). The analysis of simple effects indicated appreciable differences among groups after conditioning [F (2, 41) = 15.485, P < 0.01]. The post hoc analyses showed that "Mor + Mor" group (548.66 ± 22.18) significantly spent more time in the morphine-associated chamber during the posttest, when compared with "Sal + Sal" group (330.68 ± 16.95, P < 0.01) or "Sal + Mor" group (401.75 ± 10.02, P < 0.01). However, "Sal + Mor" group (401.75 ± 10.02) showed no notable preference for the morphine-associated chamber compared with "Sal + Sal" group (330.68 ± 16.95, P > 0.05).

3.2. The effect of morphine during E17-20 on locomotor activity of the chicks during the CPP test

As shown in Fig. 2, there was a significant difference in the distance travelled by the chicks during the posttest [F (2,41) = 11.013, P < 0.01]. Post-hoc analysis indicated that "Mor + Mor" group (2328.06 ± 46.40) increased their locomotion significantly, compared with "Sal + Sal" group (1991.71 ± 64.75, P < 0.01) or "Sal + Mor" group (2053.29 ± 54.42, P < 0.01). Furthermore, there was no significant difference in locomotor activity between the "Sal + Sal" group and "Sal + Mor" group (P > 0.05).

3.3. The effect of morphine during E17-20 on the level of PKCα in the IMM of the chicks after the CPP test

One-way ANOVA revealed the significant differences in the protein levels of PKCα in the membrane fractions [F (2,15) = 20.16, P < 0.01] among the groups (Fig. 3B). Post-hoc analysis indicated that the expression of PKCα in the membrane fraction of "Mor + Mor" group (0.38 ± 0.03) was lower than that of "Sal + Sal" group (0.66 ± 0.02, decrease: 42%, P < 0.01) or "Sal + Mor" group (0.61 ± 0.05, decrease: 38%, P < 0.01). However, the levels of PKCα in the IMM cytosol fractions showed opposite changes [F (2,15) = 4.943, P < 0.05] (Fig. 3B). Post-hoc analysis displayed that the expression of PKCα in the cytosol fraction of "Mor + Mor" group (1.12 ± 0.07) was higher (increase:32%, P < 0.05) than that of "Sal + Mor" group (0.76 ± 0.12). Although there was no significantly statistical difference in the level of PKCα in the cytosol fraction between the "Mor + Mor" group (1.12 ± 0.07, P > 0.05) and the "Sal + Sal" group (0.94 ± 0.03), the level of PKCα in the cytosol fractions of the "Mor + Mor" group (increase:16%) showed higher than that of the "Sal + Sal" group. We also detected the basal levels of PKCα in the IMM of chicks prenatally treated with morphine. Fig. 3C indicated that there were no significant prenatally morphine-induced alterations for basal expression of PKCα in the membrane (t_{10} = 0.183, P > 0.05) or cytosol fractions (t_{10} = 0.301, P > 0.05), when compared with the saline group.

4. Discussion

The present study employed the CPP procedure to assess the rewarding effects of morphine in chicks prenatally exposed to morphine during E17-20. We observed the obvious morphine CPP and

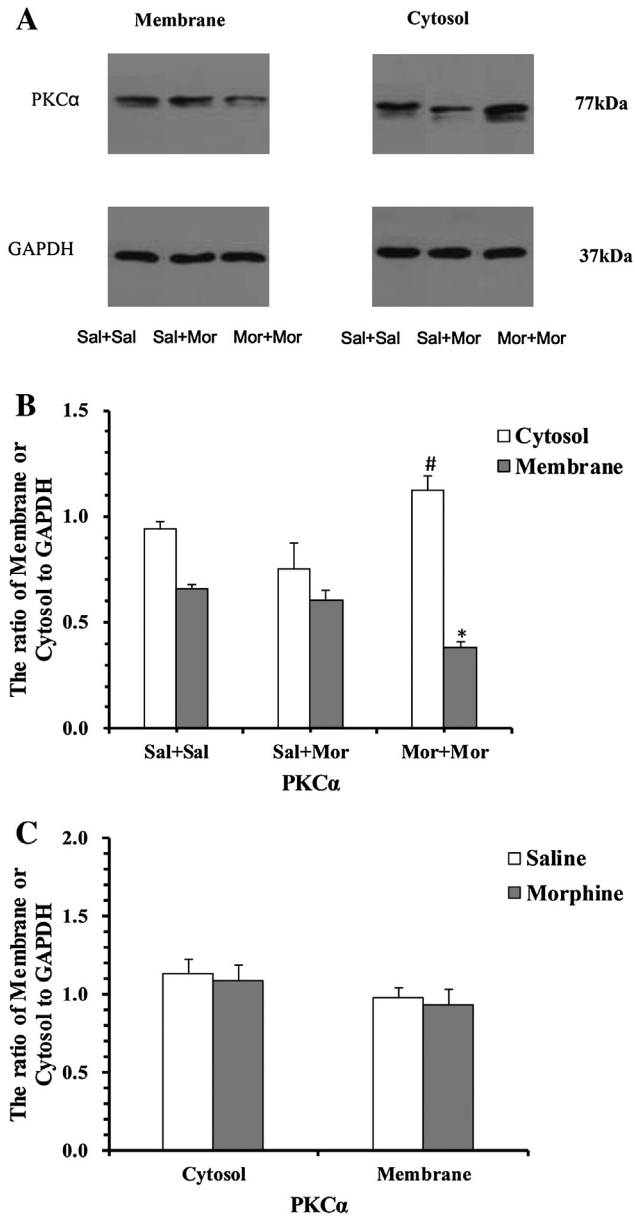

Fig. 3. The effects of prenatal morphine during E17-20 on the level of PKCα in the bilateral IMM of chicks after the CPP test. A. Representative bands from Western blots. B. The expressions of PKCα in the membrane and cytosol fractions. *, $P < 0.01$ versus "Sal + Sal" or "Sal + Mor" group. #, $P < 0.05$ versus "Sal + Sal" group. "Sal + Sal" group: prenatally saline-exposed chicks were injected intraperitoneally with saline; "Sal + Mor" group: prenatally saline-exposed chicks were injected intraperitoneally with morphine; "Mor + Mor" group: prenatally morphine-exposed chicks were injected intraperitoneally with morphine. C. The effects of prenatal morphine exposure on the basal expression of PKCα. Results are mean ± S.E.M. N = 6 per treatment group.

increased locomotor activity during the posttest phase in chicks prenatally exposed to morphine (Figs. 1 and 2). Prenatal morphine exposure significantly extended time spent in the morphine-paired environment and increased psychomotor activity at the lowest (1 mg/kg) dose, at which prenatally morphine-exposed chicks preferred the morphine-paired chamber significantly more than control group or prenatally saline-exposed chicks, as demonstrated by our previous experiments [13]. Taken together, the present results may indicate that prenatal morphine exposure during E17-20 increased the sensitivity to the rewarding effects of morphine in chicks. In addition, we found that the hatch day of morphine-exposed chicks was significantly longer than that of saline-exposed chicks (Table 1). Previous studies indicated that prenatal exposure to opiates can induce developmental delay during the neonatal and preweanling periods [9,24]. In light of this information, longer hatch day induced by prenatal morphine exposure may possibly be associated with developmental delay during the incubation stage.

It is well-known that the development of morphine addiction is mainly mediated by μ-opioid receptors [27]. After the activation of μ-opioid receptors, lots of the molecular signaling pathways are subsequently implicated in complex process, including protein kinase C [28]. Earlier reports have suggested that μ-opioid receptors could be transiently coupled to tyrosine kinase, phospholipase D (PLD) and PKCε activation in chick embryo neuron cultures [26]. Previous studies on the chronic effects of opiates on the expression of PKC isoforms in the CNS have demonstrated that μ-opioid addiction is associated with down-regulation of PKCα, but not PKCξ in the brain of heroin addicts [3,12,25], and of heroin, morphine and methadone-dependent rodents [3,45]. Interestingly, the decrease of PKCα in the CNS seems to be specific for opiates [3]. In our present studies, there was no statistical significance in the basal expression of cytosolic- and membrane-associated PKCα between prenatal saline group and prenatal morphine group (Fig. 3C). However, after the acquisition of morphine-induced CPP, the level of PKCα in the membrane was decreased whereas that in the cytosol showed an opposite trend in prenatally morphine-exposed chicks (Fig. 3A and Fig. 3B). This may suggest that there is a negative relationship between the translocation of PKCα from cytosol to membrane and the development of preference to morphine-associated chamber.

The involvement of μ-opioid receptor desensitization and/or up-regulation of the adenylyl cyclase/cAMP transduction system have been proposed as one of the best characterized adaptive molecular mechanisms underlying opiate tolerance and addiction [29,32]. There is evidence that the conventional PKCα isoform appears to be responsible for morphine-induced desensitization of μ-opioid receptors [1,16], and to regulate the activity of the adenylyl cyclase/cAMP system, such as inhibition of G_i proteins activity [45]. Post-mortem analysis of human opiate addicts has demonstrated that the level of PKCα is significantly decreased, which may be associated with the increased expression of G_i proteins [12]. These findings have shown that there may be a negative correlation between the densities of PKCα and those of Gi proteins, which is likely to aim at compensating the desensitization of the μ-opioid receptors system in the brain of opiate addicts [3,12]. Given this context, it is reasonable to postulate that the decrease of membrane-bound PKCα in IMM may appear to be associated with the acquisition of morphine CPP and with the increased locomotor activity in chicks prenatally exposed to morphine. The present study cannot provide direct evidence for the effects of prenatal morphine exposure on expression of Gi proteins. However, this still merits further investigation.

In chicks, the IMM is well-known as an important area involved in associative learning, which appears to correspond to parts of the mammalian association cortex and function as a locus for collating signals from primary sensory areas of the forebrain [15]. For example, the IMM may mediate associations between a visual image and other features (e.g., bitter taste) during the passive avoidance learning [6]. Moreover, the IMM is also known to be crucial for imprinting in chicks, which is mainly associated with storage of the visual and sound information on the imprinting object [15]. CPP employed in the present study, depends on neutral stimulus (e.g. color information) that has been associated with reward to conduct approach behavior, which is thought to be the most well-studied classical conditioning learning paradigm in drug addiction [4]. Based on information above, we inferred that IMM may be a suitable candidate for testing the rewarding effects of morphine in chicks prenatally exposed to morphine. Furthermore, there may be more of similarities in molecular basis of IMM between

reward-related learning and imprinting. Previous studies demonstrated reduced membrane-bound PKCγ expression in the IMM of chicks prenatally exposed to heroin, which had poor performances on the imprinting [49]. Our present results also support the hypothesis by showing that the loss of membrane-bound PKCα in the bilateral IMM was observed in chicks prenatally exposed to morphine, which acquired morphine CPP after conditioning. However, it should be mentioned that the IMM has strongly left lateralization effect in the passive avoidance learning [2]. Therefore, we cannot rule out the possibility that the right IMM may be entirely quiescent in the reward-related learning. To date there is still no clear evidence that lateralization has been observed for the associative drug memory in the avian system. This, however, is another meaningful issue that merits further study. Future studies on the differences in this type of learning between left and right IMM will may increase our understandings of the roles of PKC in addiction learning.

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