

The effects of morphine at different embryonic ages on memory consolidation and rewarding properties of morphine in day-old chicks

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ABSTRACT

Prenatal exposure to morphine can alter the capacities for learning and memory and the sensitivity to drugs of abuse in progeny. In the present study, we examined the effects of morphine during chick embryonic period of 5–8, 9–12, 13–16 and 17–20 on cognitive function and the sensitivities to morphine reward in the post-hatch chick, using the one-trial passive avoidance learning task and the conditioned place preference paradigm. It was observed that the injection of morphine (1 mg/kg of egg weight) during E5–8, but not in other three periods, significantly impaired intermediate- and long-term memory in one-day-old chicks. On the other hand, the chicks prenatally exposed to morphine during E17–20 remarkably not only acquired but also maintained the conditioned place preference induced by morphine. The present results suggest that there are two time-windows during development, which in the chick are around E5–8 and E17–20, when prenatal morphine exposure is likely to confer maximal risks for vulnerabilities to breakdown of memory consolidation and to morphine-induced reward in day-old chicks respectively.

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Prenatal exposure to opiates can be damaging to the development of human fetuses, leading to deficits in cognitive function and increased risk of drug dependence in exposed children [30,41]. In animal studies, considerable evidence has shown that the developing opioid system can be long-lasting changed by prenatal morphine exposure [24,34,37], which may be related to the variations in capacities for cognition [4,31] and in susceptibility to drug-taking behaviors in prenatally morphine-exposed animals [8,23,35]. However, there are no studies available on which stages of embryonic period exposed to morphine, could confer maximal risk for those neurobehavioral disorders in progeny.

Endogenous opioid systems are involved in learning and memory [1]. Opioid agonists produce memory impairments [14,16], whereas opioid antagonists enhance memory-based performance in various learning tasks [3,17]. Accordingly, alterations in capacity for cognition could be expected in offspring treated with opiates during pregnancy [4,15]. On the other hand, endogenous opioid systems also play an important role in the reward circuitry of the brain. Prenatal exposure to opiates is shown to either increase [8,21,35] or fail to alter [23,36] the rewarding properties induced by opiates or other psychoactive drugs later in life. However, those findings show the difficulties in comparing those behavioral defects pro-

duced by prenatal opiate exposure due to the differences in drug dose, schedule and route of administration, the age of the animal when tested and the type of opiate employed and so on. Behavioral studies on offspring prenatally exposed to opiates have been usually confined to rodents [40]. Unlike the rodents, the developing chicken can provide the true comparisons to be made of the direct developmental effects of prenatal influences [12], in addition to the avoidance of maternal confounds. For example, this model system has proven to be useful in the study of mechanisms of action of prenatal hypoxia [25], and administration of ethanol [22].

The main purpose of the present study was to administer intermittent injections of morphine over a wide range of embryonic development (from E5 to E20 of the 21-day incubation period) to search for the sensitive embryonic periods, which may be associated with differing vulnerability to breakdown of memory consolidation or to morphine reward in prenatally morphine-exposed chicks. After hatch, the chicks were tested on the two behavioral responses: the one-trial passive avoidance learning task (PAL) and the morphine-induced conditioned place preference (CPP).

Freshly fertilized “BAU-3” eggs (60 ± 5 g) were obtained from Beijing Agricultural University (BAU) and incubated for 21 days (E0–21) in domestic self-turning incubator (750 eggs, Beijing Hai-Jiang Incubator,) with exposure to 12 h light/dark cycles. The incubator conditions were maintained at 37.8 °C and 55–65% relative humidity. The day of birth was considered to be the day chicks emerged from their shells and designated postnatal day 0 (PND 0).

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All the experimental protocol and procedures were carried out in accordance with the requirements of the NIH guide for the care and use of Laboratory Animals (No. 8023, revised 1996).

Based on the developmental time features of opioid system in the chick embryo [6,9], we divided the embryonic period (from E5 to E20) into four stages as follows: E5–8, E9–12, E13–16 and E17–20. Morphine injections were once given every other day in each embryonic stage. Administration session was 24 h apart to allow for lower metabolism of morphine during early period of embryogenesis [27]. The present studies were conducted at a dose of 1 mg/kg of egg for morphine. This dose of morphine, as it has ever been reported that, could produce a significant inhibition of spontaneous motility (at least as early as day 5) in the early chick embryos [19]. To introduce substances, the eggs were candled and a hole that avoided membrane-bound blood vessels was drilled in the chorioallantois end of the shell. For more details, see Schrott et al. [29]. The injection volume was 20 μ l/egg. Vehicle eggs received equivalent volume of 0.9% physiological saline. Morphine was dissolved in 0.9% sterile saline.

Eggs of each embryonic age were numbered and assigned to two groups ($n=65$ /group): (1) embryonic eggs administered with morphine (1 mg/kg of egg weight, approximately 0.06 mg/egg); (2) embryonic eggs administered with physiological saline (0.9% NaCl). Control embryonic eggs in which no experimental manipulations were also assigned ($n=65$). On PND 1, chicks were trained in a one-trial passive avoidance learning (PAL) task.

Assignments and drug treatments of eggs of each embryonic age were the same with the descriptions of the experiment one (number of eggs per group = 15). After hatch, chicks received the training of the morphine CPP.

One-day-old chicks were trained using the Open University training procedure. For more details, see Gibbs et al. [10]. In the current studies, a lower concentration (20%) of methylanthranilate

(MeA, Sigma chemical Co., USA) was dissolved in absolute ethanol, since it has been reported that this concentration of MeA is close to the threshold concentration needed to produce consolidation into long-term memory [5]. Tests of memory were conducted at 30, 120 and 360 min after training. Memory retention was calculated as a percent avoidance score (i.e. the number of chicks in each group that avoided the red bead but pecked the white on test $100 \times$ /total number of the trained chicks). Each chick was trained and tested only once.

Place conditioning experiment consisted of a 6-day schedule, with three phases: pretest, conditioning and test. The place conditioning schedule and apparatus were similar to those described by our previous studies [13] with minor modifications. A 1 mg/kg dose of morphine was chosen in the present study in order to investigate the sensitivity to morphine reward in prenatally morphine-exposed chicks. After the last conditioning trial, the CPP test started 24 and 72 h.

For PAL experiment, data were analyzed using the non-parametric G-test of independence with Williams correction as recommended for the small samples [33]. As for CPP test, the time spent in the drug-paired chamber during, before and after conditioning was analyzed by a two-way ANOVA for repeated measures on one factor. The between-subject factors included "prenatal treatment" ("vehicle" versus "drug"), and the within-subject factor was "test" ("pretest" versus "test"). Posthoc tests (LSD) or the analyses of simple effects were applied to test between-group differences whenever indicated by ANOVA results. Data were expressed as mean \pm S.E.M. The level of statistical significance was set at $P < 0.05$.

As shown in Fig. 1, prenatal saline in either embryonic period did not influence memory consolidation when compared with controls. All subsequent experiments therefore employed saline groups to test the learning capacity of morphine groups. Fig. 1A shows the significant differences in percent avoidance at

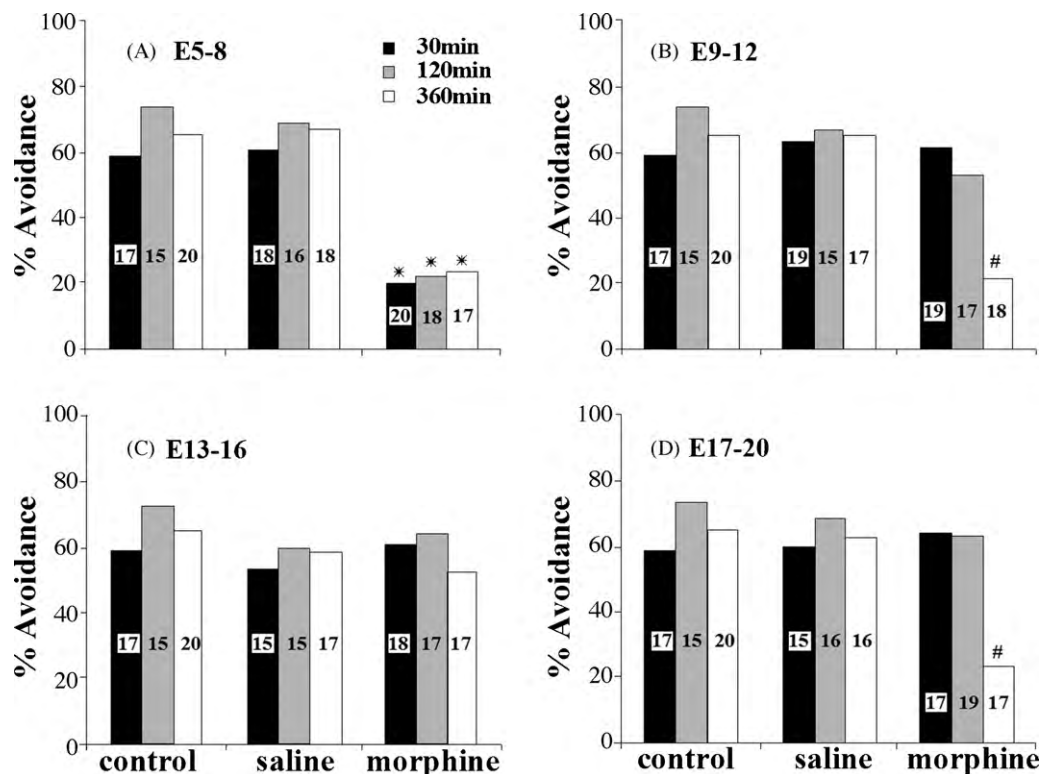


Fig. 1. The effects of prenatal morphine at E5–8, E9–12, E13–16 and E17–20 on memory consolidation in one-day-old chicks. Results are presented as percent avoidance for the passive avoidance learning tasks at 30 min, 120 min and 360 min after training. * $P < 0.01$ or # $P < 0.05$ versus prenatal saline group. Numbers of chicks in each group are presented in the relevant bars.

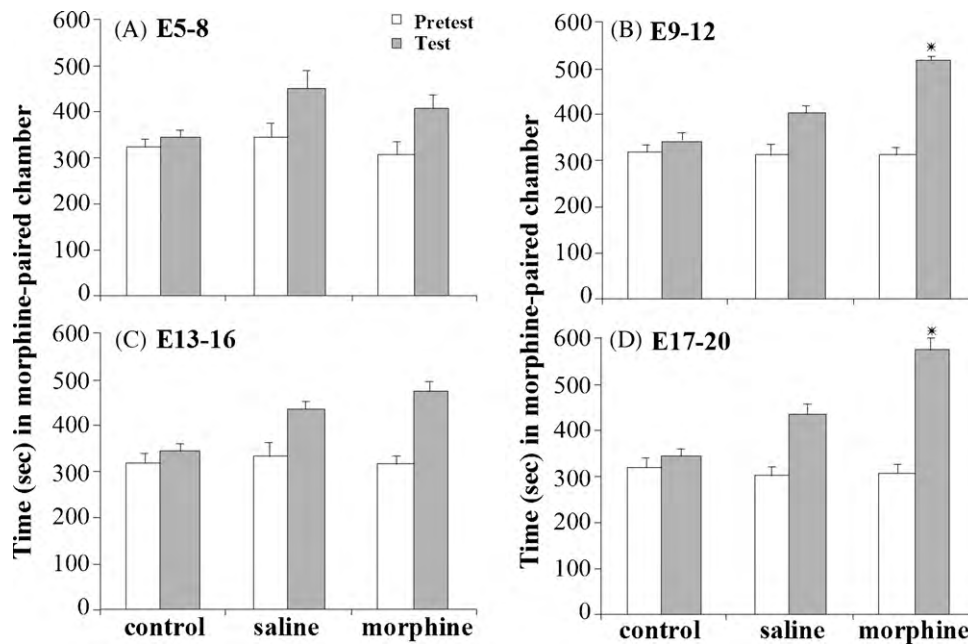


Fig. 2. The effects of prenatal morphine at E5–8, E9–12, E13–16 and E17–20 on the acquisition of morphine-induced place preference in chicks. Results are mean \pm S.E.M. * $P < 0.01$ versus control group or prenatal saline group. $n = 8–11$ per treatment group.

30 min ($G_{\text{adj}} = 6.909$, $P = 0.009$), 120 min ($G_{\text{adj}} = 7.718$, $P = 0.005$) and 360 min ($G_{\text{adj}} = 6.798$, $P = 0.009$) between chicks' saline exposure and morphine exposure at E5–8. Fig. 1B indicates that the affected chicks displayed a significant decrease in avoidance at 360 min ($G_{\text{adj}} = 6.660$, $P = 0.01$) when compared with the saline group. Similarly, a significant decrease in avoidance at 360 min ($G_{\text{adj}} = 5.267$, $P = 0.022$) was also observed in the chicks exposed to morphine at E17–20 (Fig. 1D). Taken together, the present data suggested that the chicks injected with morphine at E5–8 had impaired memory, worse than that of the same treatments at other embryonic periods.

As seen in Fig. 2B, there were a significant 'prenatal treatment \times test' interaction [$F(2, 27) = 13.392$, $P < 0.01$], and appreciable differences among groups after conditioning [$F(2, 27) = 28.12$, $P < 0.01$]. The posthoc analysis demonstrated that the chicks prenatally exposed to morphine (517.00 ± 12.20 , $P < 0.01$) remarkably preferred the morphine-paired chamber compared with those exposed to saline (405.11 ± 16.74) or controls (341.91 ± 20.17). Fig. 2D shows that there was also a significant 'prenatal treatment \times test' interaction [E17–20, $F(2, 25) = 16.602$, $P < 0.01$] among groups, and a significant difference among the groups [$F(2, 25) = 20.95$, $P < 0.01$] was observed after conditioning. The affected chicks also spent more time in the morphine-paired chamber (575.11 ± 31.38 , $P < 0.01$), compared with the saline group (436.75 ± 28.45) or controls (341.91 ± 20.17). The present data suggested that a significant morphine CPP was observed in chicks exposed to morphine at E9–12 or E17–20.

Retention of morphine CPP was tested at 72 h after the final conditioning trial. As shown in Fig. 3 (left panel), there was not a significant 'prenatal treatment \times test' interaction between the two groups [E9–12, $F(1, 19) = 4.148$, $P > 0.05$], while the right panel of Fig. 3 shows a notable 'prenatal treatment \times test' interaction between groups [E17–20, $F(1, 18) = 32.231$, $P < 0.01$]. The analysis of simple effects indicated that the morphine CPP was stronger in the chicks exposed to morphine at E17–20 [$F(1, 18) = 31.15$, $P < 0.01$], when compared with controls.

Cognitive abilities in prenatally morphine-exposed chicks were assessed using the one-trial passive avoidance learning paradigm (PAL). The present data show that prenatal administration of

morphine-induced memory impairment, which are consistent with previous results of others [4,20]. Moreover, we found that degrees of memory impairment of chicks prenatally exposed to morphine during E5–8 (Fig. 1) were more serious than that of chicks exposed to morphine during other three embryonic periods. Similar to previous studies, chick embryos injected with ethanol [22] or heroin [15] during early fetal life, indicated significant cognitive defects after hatch. Further, eggs were exposed to opiates on early gestation, the period of time during which neurogenesis and brain structures develop rapidly in the developing chick embryos [12]. Hence, these results may raise a possibility that environmental insults taking place in early development may facilitate disturbances in the development of the central nervous system such as intellectual impairment. However, these findings disagree with others [4], demonstrating that prenatal morphine exposure from E12 to E16 had significant memory impairment. Such inconsistency may be attributed to the differences in dose regimens. For example, variations in the effects of prenatal morphine could be, at least in part, due to drug withdrawal experienced by the fetus between injections [18]. The method of morphine administration used in the

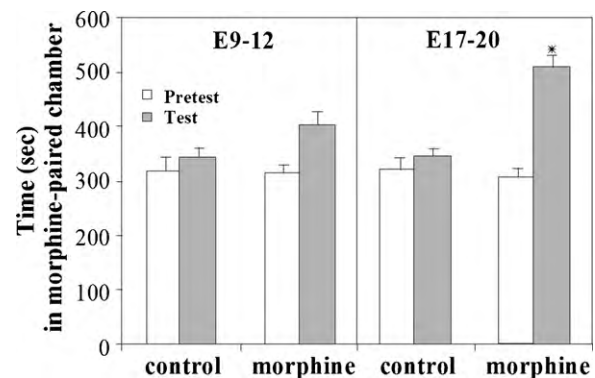


Fig. 3. The retention of morphine-induced place preference in chicks exposed to morphine at E9–12 (left panel) or E17–20 (right panel). Retention tested at 72 h after the last conditioning trial. Results are mean \pm S.E.M. * $P < 0.01$ versus control group. $n = 9–11$ per treatment group.

present study served to minimize the problem of daily withdrawal, which ensured continuous presence of the drug.

The PAL paradigm includes three overlapping stages of memory formation processes designated as short-term (STM; more than 15 min), intermediate-term (ITM; 20–55 min), and long-term (LTM; more than 55 min) memory [26]. The effects of morphine exposure during different periods of gestation on ITM and LTM formation in chicks were the particular focus of the present study. The sequential dependence of memory in chicks, that is, the inhibition of formation of one stage of memory also prevented the appearance of the later stage, has also been indicated by the present data, showing that prenatal morphine exposure at E5–8 profoundly impaired not only intermediate-term but also long-term memory. ITM formation is attributed to hyperpolarization resulting from $\text{Na}^+/\text{K}^+/\text{ATPase}$ activity [32]. Although the precise mechanism of ITM impairment induced by prenatal morphine exposure has not been described, from our results we infer that the series of rapid synaptic transients was affected by prenatal morphine exposure. The formation of LTM in the PAL paradigm involves two distinct waves of protein synthesis in the specific regions of chick brain. The first wave representing the earliest phase in LTM formation occurs within the first 2 h, which is correlated with expression of immediate early genes, *c-fos* and *c-jun* [26]. The second wave involving the synthesis of neuron/glia cell adhesion molecule occurs more than 5 h after training [28], which corresponds to the function of LTM, which is to retain and accurately retrieve the acquired information [2]. The present results indicated that memory was attenuated only at 360 min in chicks exposed to morphine at E9–12 or E17–20. Thus, from the current results it is possible to comment that prenatal morphine exposure did not influence the earliest phase in LTM formation. The specific mechanisms of impairment of LTM following prenatal morphine exposure cannot be tested by the design of the present experiments. However, of particular relevance are the expression of LTP, kinetic properties of NMDA-glutamate receptors and membrane protein phosphorylations, which are thought to be the features of LTM in the PAL paradigm [4], have been demonstrated to be significantly altered or reduced in rats prenatally exposed to morphine [20,38,39]. The specific mechanisms of impairment of LTM consolidation induced by prenatal morphine, which may be associated with the changes of protein expression and synaptic morphology [7], await further investigations.

The present study tested the rewarding effects of morphine in prenatally morphine-exposed chicks using conditioned place preference (CPP) paradigm. The present data demonstrate that exposure to morphine during different embryonic periods could produce differential sensitivity to morphine reward in offspring. We observed the obvious morphine CPP in chicks prenatally exposed to morphine at both E9–12 (Fig. 2B) and E17–20 (Fig. 2D). Further, when the retention of the morphine CPP was compared, the differences were evident indicating that the morphine CPP was still strong in chicks prenatally exposed to morphine at E17–20 (Fig. 3). Based on these results, we may conclude that prenatal morphine exposure during E17–20 could predispose morphine reward in offspring. It has been well documented that morphine induces its rewarding effects via its action on the μ -opioid receptor [11]. Moreover, some studies have demonstrated that the rewarding effects potentiated by μ -opioid receptor agonists are positively correlated with the alterations of μ -opioid receptor density induced by prenatal morphine [37]. During the late developmental stage of chick embryo, earlier studies have reported that μ sites display higher affinity [9]. Considering that the influence of morphine on opioid systems is likely dependent on the state of the receptive sites that are developing at the time of drug exposure [35], it is expected that rewarding effects of morphine observed in chicks would correlate with the long-lasting alterations of μ -opioid receptor in specific brain areas induced by prenatal morphine exposure during E17–20.

However, the present study does not provide direct evidence of functional alterations in these receptors, and it needs further investigation. It should be mentioned that the facilitated acquisition of morphine CPP is not the result of improved learning, since normal or even impaired learning induced by prenatal morphine exposure has been reported [31] or indicated in the present results. A possible explanation for the phenomenon is that the rewarding effects of morphine may predispose the chicks to perform the morphine-reinforced learning. However, this still merits further study.

In conclusion, the present study for the first time shows that there are two sensitive embryonic periods during development, which in the chick are around E5–8 and E17–20, when intermittent morphine exposure respectively leads to significant cognitive defects and to remarkably increased sensitivity to morphine reward after hatch. Future studies on neurochemistry and histology will improve our understanding of the effects of prenatal morphine on the above-mentioned behavioral defects.

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