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BRIEF COMMUNICATION

Drug Withdrawal Prior to Hatch in the Morphine Tolerant Chick Embryo

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NEWBY-SCHMIDT, M. B. AND S. NORTON. *Drug withdrawal prior to hatch in the morphine tolerant chick embryo.* PHARMACOL BIOCHEM BEHAV **18**(5) 817–820, 1983.—Morphine tolerance and dependence were produced in chick embryos by injecting 20 mg/kg into the air space of the egg daily from incubation day 12. Starting on day 16, two groups of eggs were withdrawn from morphine by either substituting water or by treating with naloxone. Chick embryo activities were monitored on incubation day 18. Activities of the embryos withdrawn from morphine did not differ from control, but naloxone injection produced a substantial increase in activity. Neither regimen of morphine withdrawal affected the hatchability of the eggs. When these chicks were 1 day old, the effect of 1 mg morphine/kg on the rate of distress vocalization was measured. The withdrawn chicks vocalized at a rate that was intermediate between that of controls and that of nonwithdrawn chicks indicating that tolerance was still present one week after the last morphine injection.

Chick Chick embryo Morphine Opiate withdrawal

THE problems associated with narcotic abuse during pregnancy have been long recognized [1]. The development of opiate tolerance and physical dependence in the immature organism is deduced mainly from the occurrence of a neonatal withdrawal syndrome shortly after birth [12]. Information concerning the time course and behavioral manifestations of narcotic dependence are largely limited to reports by pregnant addicts of increased fetal activity during maternal narcotic deprivation [1,12]. Study of the chick embryo overcomes many of the problems present in working with mammalian systems [2] and offers advantages in studies of developmental toxicity since there are no secondary effects due to maternal toxicity during the period of exposure.

The chick brain contains opiate receptors similar to those found in the mammalian brain [11]. Previous studies in this laboratory [8] have shown that opiate tolerance and physical dependence is produced in the chick embryo after four daily doses of 20 mg morphine sulfate (MS)/kg egg, starting on incubation day 12. Activity is suppressed in control embryos by morphine injection but not in embryos that receive morphine pretreatment. Tolerance is also demonstrated in the early posthatch period by the lack of effect of morphine on the rate of isolation-induced distress vocalization (DV) in the morphine-pretreated chicks. Physical dependence can be demonstrated at both ages utilizing the behavioral techniques described above and assessing the chicks' responses to naloxone.

In studies where morphine was injected into the air space of the egg up to and including incubation day 19, no effects were produced on the hatchability of the chicks or on their motor ability in the early posthatch period [8,9]. Signs of abstinence were evident after the third or fourth day posthatch. Kawahara and Sparber [6] reported that treatment of opiate tolerant chicks on incubation day 19 with naloxone caused an impairment in the chick's ability to hatch. It was of interest to see if this finding could be duplicated in our system, which differed in several respects from that study. The experiments reported in the present study were designed to examine the effects of withdrawal from morphine on the hatchability of the morphine-tolerant chick embryo and on the posthatching behavior of the chicks.

METHOD

Animals

Fertile White Leghorn chicken eggs (Larson Laboratories, Gowrie, IA) were incubated at 38° in a forced-draft incubator and turned automatically once every hour. The eggs were segregated by treatment group shortly before

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hatching (on incubation day 21). After hatching, the chicks were placed in a heated brooder with feed (Purina Starteena) and water available ad lib.

Injections

All injections into eggs were made into the air space as described previously [8]. The eggs were injected with either sterile drug solution or sterile water (vehicle) beginning on incubation day 12 and continuing up to and including incubation day 19. Volume of injection was 0.2 ml/100 g egg. Naloxone (Narcan, Endo Laboratories Inc., Garden City, NY) was injected at a volume of 0.2 ml/100 g egg, with a resultant dose of 0.8 mg/kg egg. Injections of young chicks after hatching were performed IP at a volume of 0.5 ml/100 g body weight. Morphine sulfate was dissolved in saline and was administered at a dose of 1.0 mg/kg.

Embryo Activity

The method and apparatus for measuring chick embryo activity is described in detail elsewhere [8]. Briefly, eggs were placed in nylon mesh hammocks suspended above a heated water bath and were covered with a hood that maintained proper temperature (37–38°C) and a high humidity. Movements were recorded by a Beckman dynograph on chart paper and the number of movements was counted in a blind manner after the experiment was finished. An embryo movement was considered to be any deviation of the pen that was twice the size of background. Recordings were made simultaneously on two eggs and the recording channels were allocated across the various treatment groups to control for any difference in sensitivity of the two channels.

Distress Vocalizations

Chicks were socially isolated by removing them from the incubator and placing them alone into a box (32×32×30 cm high) in a quiet room. The box was covered and after one min, the number of vocalizations was counted in the first 10 sec of each of the next nine min. Thus results were expressed as number of DV's/90 sec sample obtained over a 10 min period. Immediately after obtaining the baseline rate of DV, the chick was injected IP with morphine and returned to the incubator with the other chicks. Distress vocalizations were counted again 20 min after injection. This method is based on the method of Panksepp and co-workers [10].

Procedure

In the morphine withdrawal experiment there were five treatment groups which differed in the injections received through days 12 to 19 of incubation (Table 1). Three groups (MS tolerant, gradual withdrawal and precipitated withdrawal) received MS for four days and the two control groups received sterile water. From incubation day 16, no MS injections were given to the two withdrawal groups. Gradual-withdrawal embryos received sterile water on days 16 to 19 and the precipitated-withdrawal embryos received naloxone on days 16 and 18 and water on days 17 and 19. The naloxone control group received the same treatment as the precipitated withdrawal group on days 16 to 19. Embryo activity was measured in eggs from all treatment groups on incubation day 18. Baseline activity was recorded for 10 min, eggs were injected as shown in Table 1 on day 18, returned to the incubator and activity was recorded again 20 min after receiving the injection. No injections were made past incubation day 19. The number hatching in each group was recorded.

In order to assess the state of tolerance of the chicks in the early posthatch period, distress vocalizations were counted when the chicks were one day old. This was three days after the last injection.

TABLE 1
INJECTION SCHEDULE

Treatment	Days of Incubation				
	12–15	16	17	18	19
Water Control	H_2O	H_2O	H_2O	H_2O	H_2O
MS Tolerant	MS*	MS	MS	MS	MS
Gradual Withdrawal	MS	H_2O	H_2O	H_2O	H_2O
Precipitated withdrawal	MS	NAL†	H_2O	NAL	H_2O
Naloxone Control	H_2O	NAL	H_2O	NAL	H_2O

*All doses of morphine sulfate were 20 mg/kg egg.
†All doses of naloxone were 0.8 mg/kg egg.

Statistics

Statistical significance of differences in rate of DV or embryo activity were determined using either paired or unpaired Student's *t*-test or analysis of variance (ANOVA) [14] as indicated.

RESULTS

Embryo activities on incubation day 18 are shown in Fig. 1. Baseline activities of all treatment groups did not differ from control baseline activity. Water injections of the control and gradual-withdrawal groups did not have any effect on activity nor did morphine injection in the MS-tolerant eggs. In the precipitated abstinence group, which received its first naloxone injection two days earlier, on day 16, a second injection of naloxone caused an increase in embryo activity. In contrast, a second injection of naloxone caused a significant decrease in activity of embryos in the naloxone control group.

Hatchability of the chicks is shown in Table 2. All groups showed 75% or better hatch; there were no significant effects of either gradual or precipitated abstinence on the hatchability of the chicks. Shown in Table 3 is the hatch from a separate experiment in which lower doses of morphine (5.0 and 10.0 mg) were employed and naloxone was administered to some of the chicks according to the schedule shown in Table 1. Though there was a trend for the MS-pretreated chicks that received naloxone on days 16 and 18 to show reduced hatch, the difference was not statistically significant ($p=0.21$, Fischer Exact Test) [3] when the 10 mg/kg group was compared with naloxone-injected controls.

Results of the DV study are shown in Fig. 2. As expected from previous experiments [8] and from the work of others [10], the controls responded to 1 mg/kg MS with a great decrease in their rate of DV. The MS tolerant chicks were resistant to this effect of morphine. The naloxone controls responded to the MS injection in a manner similar to controls; hence the two prehatch injections of naloxone did not alter their sensitivity to morphine in the posthatch period.

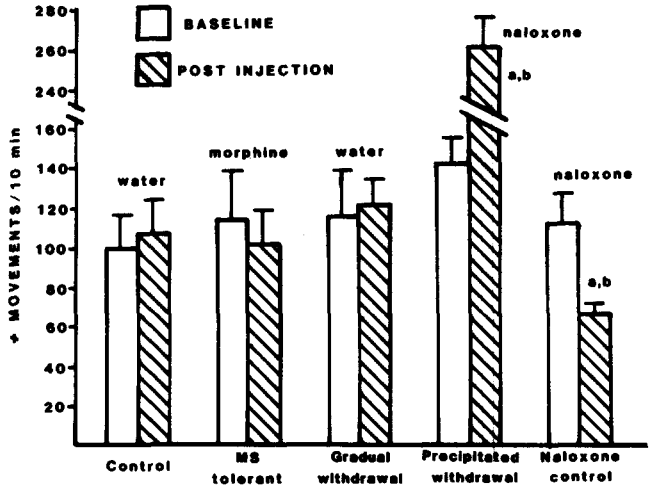

FIG. 1. Chick embryo activity on incubation day 18. Eggs were pretreated during incubation days 12 to 17 according to the schedule shown in Table 1. On day 18, baseline embryo activity was recorded for 10 min prior to injection of the agent indicated above. Post-injection activity was recorded 20 min later. Values represent mean±SE, N=5 eggs/group except for MS tolerant, N=4 eggs/group. [a]Significantly different from respective baseline, paired Student's t-test, $p<0.05$. [b]Significantly different from respective control, unpaired Student's t-test, $p<0.05$.

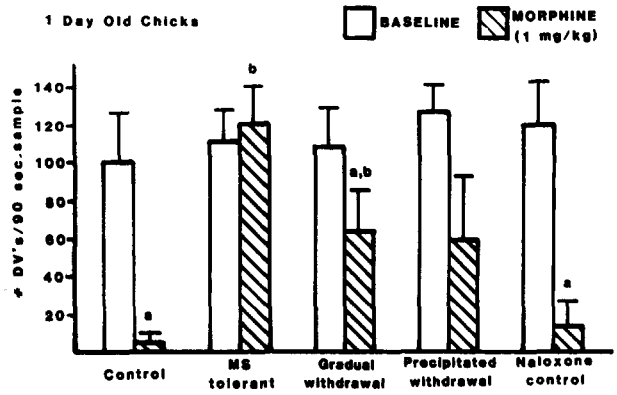

FIG. 2. Distress vocalizations of one day old chicks. Eggs were treated on incubation days 12 to 19 according to the schedule shown on Table 1. Injections were stopped after incubation day 19 and eggs were allowed to hatch. One day old chicks were socially isolated for one min and the number of DV's were counted in the first 10 sec of each of the next 9 min. Immediately after obtaining the baseline rate of DV, chicks were injected with 1 mg MS/kg and rate of DV was determined again 20 min later. Values represent mean±SE, N=5 chicks/group except for naloxone control, N=4 chicks/group. [a]Significantly different from respective baseline DV, paired Student's t-test, $p<0.05$. [b]Significantly different from respective control, unpaired Student's t-test, $p<0.05$.

TABLE 2

HATCHABILITY OF EGGS

Treatment Group	No. Hatched/ No. Eggs
Control	8/8
MS Tolerant	6/8
Gradual Withdrawal	6/8
Precipitated Withdrawal	7/8
Naloxone Control	7/8

Eggs were injected from incubation days 12 to 19 according to the schedule shown on Table 1. Injections were stopped after day 19 and eggs were allowed to hatch.

TABLE 3

HATCHABILITY OF EGGS AT LOWER DOSES OF MORPHINE

Treatment		
Morphine Dose (mg/kg)	+ Naloxone	No. Hatched/ No. Eggs
0.0	No	8/8
5.0	No	7/8
10.0	No	8/8
0.0	Yes	7/8
5.0	Yes	6/8
10.0	Yes	4/7

Eggs were injected from incubation days 12 to 15 with either sterile water or MS at the dose indicated. The eggs either received MS or water from days 16 to 19 or 0.8 mg naloxone/kg on days 16 and 18 and water on days 17 and 19. Injections were stopped after day 19 and eggs were allowed to hatch.

The two withdrawal groups showed responses intermediate between the control and MS-tolerant groups. The gradual withdrawal chicks showed significant decrease in rate of DV after morphine injection. Neither withdrawal group had a rate of DV after morphine that was significantly different from the MS tolerant group. Hence there appears to be some residual tolerance in the withdrawn groups, but the tolerance is not as complete as in the MS tolerant group of chicks.

DISCUSSION

The results of the embryo activity study agree with results of previous experiments [8] in which embryo activity was measured on day 16. In that study, 20 mg/kg MS caused a greater than 50% decrease in embryo activity of controls, but embryos that had received MS from days 12–15 were completely tolerant to this effect. Naloxone treatment caused a slight decrease in activity of controls and a great increase in activity of MS-pretreated embryos.

Morphine-dependent chick embryos react to the presence of a narcotic antagonist by greatly increasing activity (Fig. 1). One might expect therefore that an embryo undergoing spontaneous withdrawal would show an increased rate of baseline activity. This was not the case for those embryos in the gradual withdrawal group. The absence of any effect of termination of MS treatment on baseline activity three days later, combined with the evidence of MS tolerance in the early posthatch period suggest that a characteristic withdrawal syndrome was not produced in ovo by this procedure. In the precipitated withdrawal group, the baseline ac-

tivity was higher than control, though the increase was not statistically significant. Naloxone injection of morphine-dependent embryos produces at least a transient state of opiate withdrawal.

Withdrawal from narcotics in utero is considered to be harmful to the mammalian fetus especially when this occurs later in gestation [13]. Whether or not this is due to a direct effect on the fetus is not known. In order to rule out effects due to maternal toxicity, Kuwahara and Sparber [6] used the chick embryo to corroborate the detrimental effects that were produced by precipitated abstinence in utero on rat offspring [7]. Their system involved a single injection of a long-acting opiate agonist on incubation day 3 and recording of chick embryo activity on day 19. They found reduced baseline activity in the narcotic-treated chicks and naloxone injection (1.7 mg/kg) increased activity back to the baseline activity of controls. Very high doses of naloxone (10 mg/kg) resulted in a reduced hatch, an effect that is probably related to impaired performance of the proper motions required for successful hatch and has little relevance to the question of deleterious effects of naloxone on the opiate-dependent rat fetus. In our system there were no effects of withdrawal on hatchability of chicks pretreated with various doses of MS (Tables 2 and 4).

Injection of MS into the air sac above a chick embryo can produce tolerance and dependence. In previous experiments [8] and the experiments reported here (Fig. 1), doses of 20 mg MS/kg egg were administered. Measurement of embryo activity showed tolerance to the activity-depressing effects of a subsequent injection of MS at the same dose level (20 mg/kg) as well as evidence of withdrawal after naloxone injection. The withdrawn chicks received their last dose of MS on incubation day 15. Twenty-four hours after hatching (one week later) these chicks are still tolerant to a challenge dose of 1 mg MS/kg IP (Fig. 2). The presence of tolerance may be due to the high dose of MS administered to the embryo. If the 20 mg/kg dose of morphine is far greater than the amount that the chick embryo is capable of metabolizing in 24 hours, then there would tend to be an accumulation of morphine within the egg over the four days of injection. The result would be a gradual tapering off of the level of morphine exposure to the chick after termination of morphine injections and hence no severe abstinence syndrome would result. This was the rationale for determining the hatchability of chicks that received lower doses of morphine shown in Table 3. Though there was no statistically significant effect of naloxone treatment on the chicks' ability to hatch, the reduced hatch in the 10 mg/kg group suggests that this dose of morphine may be more appropriate for study of withdrawal in ovo.

One question raised by the data in this report is that of the rate of MS metabolism in the chick embryo. The adult chicken metabolizes morphine primarily via sulfate conjugation with some formation of the glucuronide conjugate as well [5]. It is not known whether sulfate conjugation is also the route of MS metabolism in the chick embryo, but since sulfate conjugation is generally higher than glucuronidation in the mammalian fetus, this may hold true for the chick embryo as well [4]. There is very little information available regarding sulfate conjugation of xenobiotics in the developing organism.

It would be desirable to obtain more information about the metabolism of MS by the chick embryo. Without this knowledge, one cannot determine whether the long-lasting tolerance to MS after treatment for several days at 20 mg/kg is due to accumulation of MS in the egg or whether there has been an alteration of opiate receptor sensitivity so that tolerance exists in the absence of morphine.

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