Pharmacology Biochemistry & Behavior, Vol. 34, pp. 705–709. © Pergamon Press plc, 1989. Printed in the U.S.A. 0091-3057/89 $3.00 + .00

Evidence of Single Dose Opioid Dependence in 12- to 14-Day-Old Chicken Embryos

M. E. BRONSON[1] AND S. B. SPARBER

University of Minnesota, Department of Pharmacology
Millard Hall, Minneapolis, MN 55455

Received 12 September 1988

BRONSON, M. E. AND S. B. SPARBER. *Evidence of single dose opioid dependence in 12- to 14-day-old chicken embryos.* PHARMACOL BIOCHEM BEHAV **34**(4) 705–709, 1989.—We have previously reported that chicken embryos injected with a single dose of methadone (Meth) on day 3, 7 or 11 of embryogenesis fail to show dependence on day 14, measured as a significant overshoot in motility above baseline after challenge with the opioid antagonist naloxone (Nx). Constant infusion of Meth from day 7 to 14 also failed to produce evidence of dependence on day 14. To address the question of whether the 14-day-old embryo is capable of expressing withdrawal, isobutylmethylxanthine (IBMX), a compound that produces quasiopioid withdrawal, was injected directly into the embryo, resulting in a significant increase in motility. To determine whether the 14-day-old embryo could also express true opioid withdrawal, the embryos were injected with various doses of Meth or morphine (Morph), followed at different time intervals by injections of varying doses of Nx. A high dose of Morph followed 24 hours later by a low dose of Nx produced evidence of withdrawal, as did a low dose of Meth followed 1 hour later by a higher dose of Nx. U50488H, a selective kappa agonist, had no effect on motility in the 14-day-old embryo, suggesting that the decrease in motility seen after Meth was not mediated by a kappa receptor. Pretreatment with the irreversible mu antagonist, beta-funaltrexamine (B-FNA), blocked the decrease in motility seen after Meth and also prevented the overshoot in motility when Nx was given 1 hour post-Meth. We were also able to demonstrate dependence/withdrawal in the 12-day-old embryo, but higher doses of both Meth and Nx were required. Pretreatment with Morph on day 9 led to decreased motility on day 10, but apparently failed to induce dependence as a broad range of doses of Nx did not increase motility above baseline. IBMX also failed to increase motility on day 10, leading us to conclude that there may be a sensitive period during embryogenesis before which this sign of opioid withdrawal cannot be expressed. Because opioid receptors are present and functional even prior to this stage of development, the failure to induce a behavioral sign of withdrawal or quasi-opioid withdrawal on day 10 suggests that opioid detoxification without threat of withdrawal-induced morbidity or mortality may be attainable at early stages of development.

Acute opioid dependence Opioid withdrawal Methadone Morphine Chicken embryos
Isobutylmethylxanthine Quasiopioid withdrawal

WE have previously reported that withdrawal can be precipitated in chicken embryos on day 17 (22) or later (12) during embryogenesis if the fertilized egg has received a single injection of the long-acting opioid N-desmethyl-l-alpha-acetylmethadol (NLAAM). Attempts to precipitate withdrawal on day 14, however, have been unsuccessful with both NLAAM and a shorter-acting opioid, methadone, which is the drug of choice for humans in opioid maintenance programs. The current research was undertaken to further characterize opioid dependence and withdrawal in the embryo, and to determine if there is a sensitive period during embryogenesis for the development of opioid dependence and the subsequent expression of withdrawal.

Our initial studies concentrated on our inability to precipitate withdrawal in the 14-day-old embryo following exposure to either NLAAM or Meth. Because radiolabeled Meth can be shown to be rapidly taken up into 14-day-old embryonic brain, and eliminated with a half-life of about 2.5 hours (22), we speculated that a single injection of this opioid 3–7 days earlier may not have a sufficiently long duration of action to induce and sustain dependence, as was observed earlier with NLAAM, when withdrawal was precipitated on day 17. In the case of NLAAM, the dose(s) of Nx used on day 14 may have been too low, which may also have been the case with Meth-exposed 14-day-old embryos. Another possibility was that the 14-day-old chick embryo is not sufficiently developed to respond with an increase in motility, above that of its basal level, at this stage.

To address the latter speculation, we administered 3-isobutyl-1-methylxanthine (IBMX), a nonopioid compound that is able to induce a quasiopioid withdrawal syndrome in other species (2, 3, 10, 11). IBMX significantly increased motility, indicating that the 14-day-old embryo is capable of expressing quasiopioid withdrawal, using this parameter as the dependent variable. We then

[1]Requests for reprints should be addressed to Maureen E. Bronson, The University of North Carolina at Chapel Hill, Department of Psychology, Davie Hall, CB #3270, Chapel Hill, NC 27599-3270.

examined the relationship between various doses of Morph or Meth and Nx, administered 1 or 24 hours apart. Our results confirmed that true opioid-type dependence and withdrawal could be demonstrated in the 14-day-old chicken embryo, but that choice of a correct combination of the agonist and antagonist is critical, as is the time span between administration of the two drugs at a particular dose of either. These findings enabled us to demonstrate a similar expression of dependence/withdrawal in the 12-day-old embryo, but not in the 10-day-old, suggesting that there is a sensitive period during development before which the embryo is incapable of expressing the increase in motility which we believe is indicative of opioid withdrawal.

Because Meth and Morph have both mu and kappa activity, we tested a range of doses of the selective kappa agonist, U50488H, to determine whether the decrease in motility seen after Meth and Morph might be due to activity at a kappa receptor. This compound had no effect on motility in the 14-day-old embryo, and challenge with Nx 1 hour later was also ineffective. Further support for a mu-mediated effect of Meth and Morph was obtained by pretreating 14-day-old embryos with the selective, irreversible mu antagonist, B-FNA (20). Pretreatment on day 13 effectively blocked the decrease in motility seen 1 hour after Meth on day 14. B-FNA also prevented the overshoot in motility when Nx was injected after Meth, suggesting that Meth dependence is mediated by pharmacological effects at mu receptors in the developing chick embryo.

METHOD

Subjects

Fertile chicken eggs (Rhode Island Red × Leghorn) were obtained from the Poultry Research Division, University of Minnesota (St. Paul, MN). They were placed in a cold room maintained at 15°C for one day prior to incubation in order to synchronize embryogenesis. At the same time of day for each experiment, eggs were set in a forced-air incubator (Humidaire Hatchette, New Madison, OH) maintained at 37.5°C and 58% relative humidity. This was considered day 0 of embryogenesis.

The day before testing, all of the eggs were candled for viability and a hole for drug delivery was drilled approximately 5 mm below the air cell, close to the vitelline vessels, using a small carbide dental burr (1.2 mm). Two more holes for insertion of electrodes were drilled 180° apart, halfway down the long axis of the egg, with the drug injection hole positioned halfway between.

Drugs

IBMX (Sigma Chemical Co., St. Louis, MO) was suspended in heated 2% Pluronic® F68 (BASF Wyandotte Corp., Wyandote, MI) or dissolved in 5% hydroxypropyl-beta-cyclodextrin (Research Biochemicals, Inc., Natick, MA). Morph sulfate (S. B. Penick, New York, NY), Meth HCl (Eli Lilly, Indianapolis, IN), U50488H (Upjohn, Kalamazoo, MI) and Nx HCl (E. I. Dupont, Wilmington, DE) were dissolved in saline and B-FNA HCl (obtained from the National Institute on Drug Abuse, Research Triangle Park, NC) was dissolved in sterile water. All doses of the opioids and Nx refer to their salts.

Procedure

Day 14.

IBMX. IBMX was dissolved in 2% pluronic and injected directly into the embryo instead of near the vitelline vessels (preliminary experiments indicated that IBMX was probably precipitating out of solution at the site of injection). A small window, approximately 2 cm by 2 cm, was chipped in the shell halfway between the electrode holes, starting approximately 1 cm below the air sac (19). A 25 μl volume of IBMX suspension (5 mg/kg egg, i.e., 0.25 mg/embryo) was then injected into the embryo with a 22 mm, 25 gauge needle. During motility recording, the window was covered with heated parafilm.

Opioids. All compounds were injected via the injection hole in the shell. In the first study, Meth or Morph (20 mg/kg egg) was given on day 13, 24 hours prior to challenge with Nx (5 mg/kg egg) on day 14. In addition, Meth (1, 2.5, 10 or 20 mg/kg egg) was given on day 14, one hour prior to challenge with Nx (5 or 10 mg/kg egg). U50488H (0.2, 0.4, 0.6, 0.8, 1 or 2 mg/kg) was administered on day 14 and Nx (20 mg/kg egg) was injected 1 hour later. In an attempt to block dependence/withdrawal, the μ selective irreversible antagonist, B-FNA (20 mg/kg egg) (20), or its vehicle was given on day 13, 24 hours prior to injection of 2.5 mg Meth/kg egg. Nx (10 mg/kg egg) challenge was made 1 hour post-Meth as in the previous experiment.

Day 12. In the 12-day-old embryos saline or Meth (2.5 or 10 mg/kg egg) was given 1 hour prior to saline or Nx (10 or 40 mg/kg egg).

Day 10.

IBMX. For the 10-day-old embryos, a relatively new compound was obtained that dissolves IBMX; therefore, the injection was made via the injection hole in the shell described above. Doses of IBMX were 0.01, 0.1, 1, 2, 4 and 8 mg/kg egg, with corresponding injection volumes (20, 40 or 80 μl) for the vehicle controls.

Opioids. In the 10-day-old embryos, Meth (2.5 mg/kg egg) was given 1 hour prior to 10 mg Nx/kg egg. Because a relatively high dose of both Meth and Nx was required to demonstrate dependence/withdrawal on day 12, and because other investigators have had difficulty producing and sustaining opioid dependence with Meth (13,23), we also used morphine following the 24-hour procedure described for day 14. Saline or Morph (20 mg/kg egg) was given on day 9, 24 hours prior to Nx (5, 20 or 40 mg/kg egg) or saline on day 10.

Motility Recording

Two platinum wire electrodes were used to conduct the electrical potential produced by embryonic movements (7,14). The electrodes extended 4 mm from the end of the barrels of 1 ml plastic disposable syringes that were held in place by micromanipulators and enclosed in a table top incubator (Forma Scientific Inc., Marietta, OH) maintained at 37°C. The electrodes were inserted approximately 2 mm into the egg. Embryonic movements were recorded on a Gilson polygraph recorder, chopper #ECG-20 (Gilson Medical Electronics, Middleton, WI), which was calibrated daily such that a 0.5 mV internal calibration signal produced a 4 cm pen deflection.

Motility Analysis

Only 2-second intervals during which the pen made at least one deflection greater than 1 cm above the baseline record of a nonviable embryo were counted as epochs of motility. The average number of epochs per minute was then used for data analysis.

Fourteen-day-old embryos.

IBMX. Baseline motility was recorded for 5 minutes, after which IBMX or its vehicle was injected. Levels of motility were again recorded for 5 minutes, starting one minute after injection.

Opioids. Baseline motility (effects of the opioid, 1 or 24 hours following injection) was determined on day 14 for 5 minutes; Nx was injected and motility was then measured for an additional 5

TABLE 1

EFFECTS OF IBMX (5 mg/kg) ON DAY 14

	Epochs per Minute (mean ± S.E.)	
	Baseline	Treatment
2% Pluronic (n = 9)	10.6 ± 2.4	9.4 ± 2.0
IBMX (n = 10)	10.2 ± 1.1	14.8 ± 1.2*†

*Significantly different from baseline, $p<0.05$.
†Significantly different from saline after pluronic, $p<0.05$.
Vehicle is 2% Pluronic. Baseline motility was recorded for 5 minutes before injection of either IBMX or Pluronic. Motility was then recorded for an additional 5 minutes, starting 1 minute after injection.

minutes, starting one minute after injection of Nx.

Ten- and twelve-day-old-embryos. It was noted in the 14-day-old embryo studies that baseline motility was relatively stable over the 5-minute recording period. In addition, the effect of either IBMX or Nx was very rapid, i.e., within 2 minutes. Thus, in the 10- and 12-day-old embryo studies, baseline motility was recorded for 2 minutes; Nx or saline was then injected and motility was recorded for an additional 2 minutes, starting 2 minutes after injection. It was necessary to include a saline-saline group for these experiments because a prior study with 12-day-old embryos revealed an apparent "injection effect" on motility. In the case of IBMX, baseline motility was recorded for 2 minutes, after which IBMX or vehicle was injected and an additional 2 minutes were recorded, starting 2 minutes postinjection.

Data Analysis

Motility data were analyzed by repeated measures and one-factor ANOVAS, followed by Dunnett's or paired *t*-tests where appropriate.

RESULTS

Day 14

IBMX. Table 1 shows that IBMX, but not its vehicle (2% Pluronic), significantly increased motility above baseline as measured just prior to injection [Repeated Measures ANOVA, $F(1,16) = 4.592$, $p<0.05$]. Baseline motility was not significantly different in the 2 groups; however, it should be pointed out that the baseline motility in this group of embryos was somewhat lower than that of 14-day-old embryos in the other studies. This difference could be due to a difference in flock and/or season, or it might be due to slight differences in the stage (age) of the embryo, all of which argue for the importance of using contemporaneous controls each time an experiment of this type is carried out.

Twenty-four-hour opioid pretreatment. Morph or Meth injected on day 13 did not significantly alter baseline motility on day 14, compared to saline-injected controls (Table 2). Only in the Morph-pretreated group receiving Nx was there a significant increase in motility above baseline, as indicated by a significant treatment by repeated measures interaction, $F(1,10) = 11.961$, $p<0.01$. Thus, in a 24-hour time span, Morph, but not Meth, was able to produce and sustain demonstrable opioid dependence under these experimental conditions.

One-hour studies.

Methadone. Meth (2.5, 10 or 20 mg/kg egg) decreased motility approximately 50% on day 14, while 1 mg Meth/kg egg had no effect. Challenge with Nx (5 mg/kg egg) reversed the decrease in motility in all instances, but did not produce the overshoot in motility which, according to our data with IBMX, is characteristic of withdrawal in ova. Because the dose of Nx may not have been high enough to precipitate withdrawal in the above study, the experiment was repeated with 2.5 and 20 mg Meth/kg egg, followed by 10 mg Nx/kg egg) 1 hour later (Table 3). Repeated measures ANOVA revealed a significant effect of Nx injection, $F(2,17) = 35.157$, $p<0.001$, which was apparent only in the Meth pretreated groups as indicated by a significant treatment by repeated measures interaction, $F(2,17) = 12.934$, $p<0.0005$. However, a Dunnett's test revealed that only the Meth 2.5 mg/kg egg group experienced withdrawal, indicated by significantly greater motility after injection of Nx when compared to saline controls after Nx injection.

U50488H. In the range of doses tested (0.2 to 2 mg/kg), U50488H had no effect on motility. Injection of Nx (20 mg/kg) 1 hour later was also ineffective, i.e., there was no evidence of dependence/withdrawal as we are measuring it.

B-FNA. B-FNA, administered on day 13, blocked the decrease in motility otherwise seen with Meth (2.5 mg/kg) on day 14. The overshoot in motility observed when Nx is given 1 hour after Meth was also blocked.

Day 12

The same dose combination of agonist-antagonist (Meth 2.5

TABLE 2

EFFECTS OF Nx (5 mg/kg EGG) ON DAY 14, 24 HOURS POST-METH OR MORPH (20 mg/kg EGG)

	Epochs per Minute (mean ± S.E.)	
	Baseline	Post-Nx
Saline (n = 11)	7.6 ± 1.3	9.5 ± 1.1
Meth (n = 8)	8.5 ± 1.4	7.2 ± 1.4
Saline (n = 6)	14.0 ± 1.6	13.5 ± 2.4
Morph (n = 6)	12.1 ± 1.8	18.5 ± 2.0*

*Significantly different from baseline, $p<0.05$.
Baseline motility was recorded for 5 minutes. Nx injection was made immediately after baseline recording and 5 minutes of motility were monitored starting 1 minute afterward. Because the Morph experiment was done on a different group of embryos than the Meth study, the results have been analyzed separately.

TABLE 3

EFFECTS OF Nx (10 mg/kg EGG) ON DAY 14, 1 HOUR POST-METH

	Epochs per Minute (mean ± S.E.)	
	Baseline	Post-Nx
Saline (n = 7)	6.1 ± 1.4	15.5 ± 2
Meth 2.5 mg/kg egg (n = 7)	9.7 ± 1.7*	22.1 ± 2†
Meth 20 mg/kg egg (n = 6)	6.9 ± 1.4*	14.6 ± 2.8

*Significantly different from saline baseline, $p<0.01$.
†Significantly different from saline after naloxone, $p<0.05$.
Other details are the same as in Table 2.

TABLE 4

EFFECTS OF Nx (40 mg/kg EGG) OR SALINE ON DAY 12, 1 HOUR POST METH (10 mg/kg EGG) OR SALINE

	Epochs per Minute (mean ± S.E.)	
	Baseline	Treatment
Saline-Saline, Saline-Nx (n = 13)	30.0 ± 1.6	18.6 ± 2
Meth-Saline (n = 7)	8.5 ± 1.7*	3.3 ± 0.6
Meth-Nx (n = 7)	7.8 ± 3.5*	27.0 ± 2.5†

*Significantly different from saline, $p<0.001$.

†Significantly different from saline after saline or naloxone, $p<0.005$.

Baseline motility was recorded for 2 minutes. Nx or saline was injected immediately after baseline recording and 2 additional minutes of motility were monitored starting 2 minutes after injection.

mg/kg egg, followed 1 hour later by Nx 10 mg/kg egg) that produced dependence/withdrawal on day 14, did not do so on day 12. A higher dose of Meth (10 mg/kg egg) was needed to significantly decrease motility 1 hour later as compared to saline controls [ANOVA, $F(1,25) = 79.237$, $p<0.0001$]. In the methadone-pretreated group, motility was further decreased after the saline injection, and in the saline-pretreated groups, both saline and Nx were followed by decreased motility, suggesting an acute injection effect. Because the saline-pretreated groups were not different during recording of baseline motility or after injection of either saline or Nx, the saline groups were combined and compared to the Meth-saline and Meth-Nx groups (Table 4). There was a significant repeated measures by treatment interaction, $F(2,24) = 30.52$, $p<0.0001$; however, a Dunnett's test revealed that only the Meth-treated embryos that later got Nx experienced withdrawal, as evidenced by significantly greater motility after injection of Nx when compared to the combined controls.

Day 10

On day 10, as on day 12, Meth (2.5 mg/kg egg) failed to decrease motility and challenge with 10 mg Nx/kg did not alter motility. In contrast to 14-day-old embryos, motility in Morph-pretreated embryos was still depressed 24 hours post-Morph [ANOVA, $F(1,25) = 15.843$, $p<0.0005$]. Nx (5, 20 or 40 mg/kg egg), failed to alter motility in either saline- or Morph-pretreated embryos. IBMX was then administered to another group of embryos to determine if 10-day-old embryos are capable of expressing withdrawal. In doses ranging from 0.01–8 mg/kg, IBMX failed to produce an increase in motility.

DISCUSSION

One of the purposes of this study was to determine if the 14-day-old chicken embryo is capable of expressing opioid-type withdrawal, and, if so, whether true opioid dependence could be produced at this time of embryogenesis or earlier. Our initial experiments with IBMX demonstrated that the mechanisms for the expression of withdrawal are present at least as early as day 14. However, although opioid receptors are present in this species by day 4 of embryogenesis (6), the possibility still existed that a 14-day-old embryo cannot undergo the adaptive processes necessary for the development of opioid dependence.

In addition, there was a time factor of potential importance. We did not know how many hours it would take for absorption and distribution of the opioids in amounts sufficient to produce dependence and yet not allow extensive metabolism of the opioid before Nx challenge. By administering Morph (20 mg/kg) 24 hours before Nx (5 mg/kg egg), we were able to demonstrate acute dependence in the 14-day-old embryo. A possible explanation for our inability to produce dependence with the same dose of Meth in the 24-hour study, is that Meth is 10 times less potent in binding studies (^{3}H-etorphine displacement) compared to Morph (5). Thus, Morph would presumably be more potent in producing the adaptive changes which occur during development of opioid dependence. On the other hand, the difference in effect between Meth and Morph may have been due to a difference in general disposition of the two drugs (18). For example, we have found that Meth has a very short half-life in brain in the 14-day-old chick embryo (22).

The fact that we could not demonstrate withdrawal in 14-day-old embryos injected 1 hour earlier with 20 mg Meth/kg egg, but could do so after 2.5 mg/kg egg, suggests that the dose of Nx was too low to displace sufficient quantities of Meth after the high dose. Single dose dependence studies in our laboratory (4,17) have demonstrated the importance of utilizing an appropriate dose of Nx to precipitate withdrawal in mature rats. In those studies, a low dose of the antagonist merely reversed the acute operant behavioral suppression caused by a dose of Morph given a few hours earlier, whereas doubling the dose of Nx completely suppressed responding as a result of precipitated withdrawal. Another possibility is that the high dose of Meth used in the current study depressed motility by opioid-specific actions (i.e., were Nx-reversible), but also blocked an increase in motility above baseline by some nonspecific effect.

Morph and Meth show a different time course of effects but they are qualitatively similar in that they both induce dependence, which can be demonstrated under appropriate conditions. The fact that U50488H, a selective kappa agonist, had no effect on motility, either immediately or 1 hour later, would suggest that the motility-decreasing effects of Morph and Meth are due to activity at mu rather than kappa receptors. This is further supported by the fact that B-FNA, an irreversible mu antagonist, blocked the decrease in motility seen when Meth 2.5 mg/kg egg is administered one day later, attesting to its alkylating capacity in this species as well.

Our findings in the 12- and 14-day-old chicken embryo are very similar to those reported by Umans and Szeto (25) in the fetal lamb. They report that moderate to high doses of Morph decreased fetal movement and that short-term exposure to the opioid resulted in acute dependence as measured by the ability of Nx to precipitate withdrawal, one of the signs of which was significantly increased body movement. Our finding that B-FNA also blocked the overshoot in motility when Nx is administered 1 hour after Meth, suggests that the phenomenon we are observing in the chick embryo is indeed opioid withdrawal.

Our data from the 12-day-old embryos confirmed that this age group expresses withdrawal in a manner similar to older embryos, i.e., an increase in motility. Ten-day-old embryos, on the other hand, while susceptible to the motility-decreasing effects of Morph, appear to be at a sensitive time of development in which either they are incapable of becoming dependent, or they cannot express withdrawal. Our data demonstrate the plausibility of studying the consequences of opioid exposure and/or opioid-type withdrawal during mid-embryonic development of the chicken. The present experiments also point to the need to do dose-ranging experiments in order to obtain the correct combination of agonists and antagonists. Our results with the chicken embryo add to the list of species which are able to develop acute opioid dependence, manifest as withdrawal upon challenge with Nx a short while (i.e., hours) after a single injection of the opioid [Martin and Eades (16),

dog; Meyer and Sparber (17), rat; Stevens and Klemm (24), Ritzmann (21), mouse; Umans and Szeto (25), fetal lamb; Jones (8,9), opioid naive humans; Bickel *et al.* (1), ex-opioid addicts]. With the exception of Umans and Szeto's studies in the fetal lamb and our work with the chicken embryo, all of the acute opioid dependence research has been done in the adult.

Of potential clinical interest is our finding that the 10-day-old embryo, while susceptible to the motility-decreasing effects of Morph, does not appear to become dependent or express withdrawal. Although opioid detoxification has proven to be very dangerous to the fetus in advanced pregnancy (26), it is possible that detoxification could be achieved early during embryogenesis, without the threat of withdrawal-induced fetal morbidity or mortality. It should be pointed out, however, that our methods for measuring the phenomenon of withdrawal (e.g., motility monitoring) may not be sensitive enough to detect more subtle signs of withdrawal in the young embryo. Further research in this area is clearly warranted.

ACKNOWLEDGEMENTS

Supported in part by USPHS grants DA 01880 and T32 DA07097. Technical assistance of G. F. Seran is appreciated.

REFERENCES

1. Bickel, W. K.; Stitzer, M. L.; Liebson, I. A.; Bigelow, G. E. Acute physical dependence in man: effects of naloxone after brief morphine exposure. J. Pharmacol. Exp. Ther. 244:126–132; 1988.
2. Collier, H. O. J.; Cuthbert, N. J.; Francis, D. L. Character and meaning of quasi-morphine withdrawal phenomena elicited by methylxanthines. Fed. Proc. 40:1513–1518; 1981.
3. Collier, H. O. J.; Francis, D. L.; Henderson, G.; Schneider, C. Quasi morphine-abstinence syndrome. Nature 249:471–473; 1974.
4. Gellert, V. F.; Sparber, S. B. A comparison of the effects of naloxone upon body weight loss and suppression of fixed-ratio operant behavior in morphine-dependent rats. J. Pharmacol. Exp. Ther. 201:44–54; 1977.
5. Gibson, D. A.; Vernadakis, A. (^{3}H)-etorphine binding activity in the early chick embryos: brain and body tissue. Dev. Brain Res. 4:23–29; 1982.
6. Gibson, D. A.; Vernadakis, A. Critical period for LAAM in the chick embryo: toxicity and altered opiate receptor binding. Dev. Brain Res. 8:61–69; 1983.
7. Jackson, H.; Rubel, E. W. Ontogeny of behavioral responsiveness to sound in the chick embryo as indicated by electrical recordings of motility. J. Comp. Physiol. Psychol. 92:682–696; 1978.
8. Jones, R. T. Dependence in non-addict humans after a single dose of Morph. In: Way, E. L., ed. Endogenous and exogenous opiate agonists and antagonists. New York: Pergamon Press; 1979:557–560.
9. Jones, R. T. Caffeine enhances morphine dependence in humans. In: Takagi, H.; Simon, E., eds. Advances in endogenous and exogenous opioids. New York: Elsevier; 1981:472–474.
10. Kleven, M. S.; Sparber, S. B. Modification of quasi-morphine withdrawal with serotonin agonists and antagonists: evidence for a role of serotonin in the expression of opiate withdrawal. Psychopharmacology (Berlin) 98:231–235; 1989.
11. Kleven, M. S.; Sparber, S. B. Morphine blocks and naloxone enhances suppression of operant behavior by low doses of 3-isobutyl-1-methylxanthine. J. Pharmacol. Exp. Ther. 248:273–277; 1989.
12. Kuwahara, M. D.; Sparber, S. B. Prenatal withdrawal from opiates interferes with hatching of otherwise viable chick fetuses. Science 212:945–947; 1981.
13. Ling, G. S. F.; Tappe, N. S.; Inturrisi, C. E. Methadone induced physical dependence in the rat. Life Sci. 34:683–690; 1984.
14. McCafferty, R. E.; Pressman, S. H.; Knisely, W. H. Recording techniques and results for amniotic and embryonic movement in situ during hen egg incubation. Biorheology 2:171–181; 1965.
15. Maderdrut, J. L.; Reitzel, J. L.; Okado, N.; Oppenheim, R. W. Behavioral analyses of opiate-mediated inhibition in the early chick embryo. Neuroscience 16:405–416; 1985.
16. Martin, W. R.; Eades, C. G. A comparison between acute and chronic physical dependence in the chronic spinal dog. J. Pharmacol. Exp. Ther. 146:385–394; 1964.
17. Meyer, D. R.; Sparber, S. B. Evidence of possible opiate dependence during the behavioral depressant action of a single dose of morphine. Life Sci. 21:1087–1094; 1977.
18. Misra, A. L. Metabolism of opiates. In: Adler, M. L.; Nanara, I.; Samanin, R., eds. Factors affecting the action of narcotics. New York: Raven Press; 1978:297–343.
19. Oppenheim, R. W.; Levin, H. L.; Harth, M. S. An investigation of various egg-opening techniques for use in avian behavioral embryology. Dev. Psychobiol. 6:53–68; 1973.
20. Portoghese, P. S.; Larson, D. L.; Sayre, L. M.; Fries, D. S.; Takemori, A. E. A novel opioid receptor site directed alkylating agent with irreversible narcotic antagonistic and reversible agonistic activities. J. Med. Chem. 23:233–234; 1980.
21. Ritzmann, R. F. Opiate dependence following acute injections of morphine and naloxone: The assessment of various withdrawal signs. Pharmacol. Biochem. Behav. 14:575–577; 1981.
22. Seran, G. F.; Sparber, S. B. Metabolism of methadone by chicken embryos prevents induction of chronic opioid-type dependence after a single injection: Use of osmotic pumps for continuous infusion. Pharmacol. Biochem. Behav. 30:357–363; 1988.
23. Sparber, S. B. Developmental effects of narcotics. Neurotoxicology 7:335–348; 1986.
24. Stevens, D. R.; Klemm, W. R. Morphine-naloxone interactions: a role for nonspecific morphine excitatory effects in withdrawal. Science 205:1379–1380; 1979.
25. Umans, J. G.; Szeto, H. H. Precipiated opiate abstinence in utero. Am. J. Obstet. Gynecol. 151:441–444; 1985.
26. Zuspan, F. P.; Gumpel, J. A. Mejia-Zelaya, A.; Madden, J.; Davis, R. Fetal stress from methadone withdrawal. Am. J. Obstet. Gynecol. 122:43–47; 1975.