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Metabolism of Methadone by Chicken Embryos Prevents Induction of Chronic Opioid-Type Dependence After a Single Injection: Use of Osmotic Pumps for Continuous Infusion

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SERAN, G. F. AND S. B. SPARBER. *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**(2) 357–363, 1988.—Unlike N-desmethyl-l-α-acetylmethadol (NLAAM), a single injection of methadone (METH), near domestic chicken embryos early during development, cannot induce and sustain opioid-type dependence in the older embyro (i.e., days 14–17 of development). Injection of [^{3}H]-METH near the 14-day-old embyro, followed by differential extraction, indicated that significant quantities of unmetabolized METH gained entrance to the brain, peaking at about 1 hr and declining with a half-life of about 2.8 hr. Thus, it is probably not practical to use a single injection of this shorter-acting opioid for studying biobehavioral effects of sustained dependence and withdrawal during development in this species. Chronic infusion of METH for 7 days via an externalized Alza osmotic mini-pump resulted in significant, dose-dependent brain concentrations of [^{3}H]-METH on day 14. Even though the opioid antagonist naloxone (Nx) was unable to induce withdrawal, manifest as a significant increase in embryonic motility above that of controls, it partially reversed the depressed motility caused by the chronic infusion of [^{3}H]-METH. Since 7-day-old embryos exposed to NLAAM, at doses which can be demonstrated to produce dependence by precipitating withdrawal on day 17 of development, were also unable to express withdrawal on day 14, it is possibile that either 14-day-old chicken embryos cannot yet fully respond with an adaptive process (i.e., dependence) or its expression (i.e., withdrawal).

Embryonic toxicity Chicken embryos Osmotic mini-pump Methadone

KUWAHARA and Sparber [7] have reported that N-desmethyl-l-α-acetylmethadol (NLAAM), 2.5 mg/kg egg, injected near the 3-day-old chicken embryo (*Gallus domesticus*) is capable of producing and maintaining opioid-type dependence until hatching, at which time there was no difference in body weight between control and opioid-dependent subjects. However, spontaneous withdrawal after hatching led to retardation in body weight gain [9]. A 70% decrease in hatchability [8] resulted when NLAAM-dependent chick fetuses were challenged with a single injection of opioid antagonist naloxone (Nx, 10 mg/kg egg), on day 19 of development. Nx did not have an effect on hatchability in control embryos. NLAAM (3.4 mg/kg egg) injected on day 3 of embyrogenesis, caused a significant decrease in spontaneous motility on day 19, at which time an acute injection of Nx (1.7 mg/kg egg) reversed the depressed motility. A higher dose of Nx (5 mg/kg egg) increased motility significantly above conrols, indicating opioid withdrawal in ova, confirming the hypothesis that withdrawal during development was responsible for the decreased hatchability of otherwise viable but opioid-dependent chick embryos.

We were unsuccessful in our attempt to replicate NLAAM-induced dependence and withdrawal in the chicken embryo by injecting the shorter-acting opioid d,l-methadone (METH) in a similar manner. Injection on day 3 of embryogenesis, even at embryotoxic doses, was unable to induce and maintain opioid-type dependence later during development (e.g., day 17, unpublished observations). It is possible that the short duration of action of METH precluded our ability to demonstrate opioid-type dependence 14

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days after injection. Therefore, we injected METH at later stages of development and tested for evidence of dependence on days 14 and 17 of development. In some instances, positive controls for equivalent degrees of embryotoxicity and/or opioid dependence were accomplished by injecting NLAAM near contemporaneous groups of embryos.

To determine if significant quantities of METH reached the embryonic brain after an acute injection, we determined the distribution and apparent half-life of [^{3}H]-METH in embryonic brain after a single injection of the labeled compound near 14-day-old embryos. The results of this study indicate that it is probably the case that a single injection of METH, early during development, would not be sufficient for our need to sustain opioid-type dependence in the older embryo (i.e., day 14 or later), which metabolizes and/or eliminates this opioid rapidly.

Multiple opioid injections into domestic chicken eggs are feasible [3, 5, 15] but the increased risk of infection and the tedious nature of this protocol, coupled with the need to work out optimum dosing schedules which would not give rise to intermittent toxicity and/or withdrawal between injections, prompted a different approach. By using an externalized osmotic mini-pump (Alza, Palo Alto, CA), which continuously infused [^{3}H]-METH for 7 days, it was possible to demonstrate dose-dependent concentrations of the opioid in the 14-day-old embryonic brain. This procedure also allowed us to determine if chronic exposure to METH would produce toxicity and/or opioid-type dependence. The optimal conditions which the Alza osmotic mini-pump operates (i.e., 37°C under mammalian isotonic conditions) can easily be mimicked within an incubator by submersing the mini-pump in a solution of 0.9% NaCl and infusing the drug via a catheter connected to the egg. The results of this procedure suggest that the osmotic mini-pump is a practical alternative to multiple injections for developmental drug studies in this species.

METHOD

Subjects

Fertile chicken eggs (Rhode Island Red × Leghorn) were obtained from the Poultry Research Division, University of Minnesota (St. Paul, MN), selected for uniform size (approximately 50 g) and shape and stored in a cold room maintained at 15°C to synchronize embryogenesis. In the eggs used for motility experiments, two holes were drilled 180° apart, half-way down the long axis of the egg [4] using a small carbide dental burr (1.2 mm). A third hole, for drug delivery, was drilled half-way between the first two and approximately 2 cm below the air cell. This was the only hole drilled in eggs which were not used for motility experiments. Prior to drilling holes, the area was disinfected with a drop of 0.2% tincture of iodine and wiped with a gauze pad moistened with 70% ethanol [16]. Care was taken not to puncture the inner membrane and each hole was covered with a small piece of transparent tape (3M, St. Paul, MN).

Eggs were set in a forced-air incubator (Humidaire Hatchette, New Madison, OH) maintained at 37.5°C and 58% relative humidity at the same time of day for each experiment. This was considered day 0 of embryogenesis.

Drugs

d,l-Methadone-*o,o'*-[$^3H_2$] HCl (specific activity 8.4 Ci/mmol) obtained from the National Institute on Drug Abuse (NIDA), through Research Triangle Institute (Research Triangle Park, NC) was used in studies which required labeled METH. Radiochemical purity of the [^{3}H]-METH was determined to be greater than 95% by thin-layer chromatography on a silica gel TLC plate (EtOAc-Hxn-EtOH-NH_4OH, 60:25:14:1, R_f=0.69). Nonlabeled METH HCl was obtained from Eli Lilly (Indianapolis, IN) and NLAAM HCl from NIDA. Depending upon the experiment and final drug concentration, METH was dissolved in either 50% propylene glycol (PG), 0.9% saline or sterile water. NLAAM was dissolved in 50% PG. Nx HCl was generously donated by Endo Laboratories, Inc. (Garden City, NY) and dissolved in isotonic saline (20 mg/ml) for acute infusion into eggs. All drug solutions were prepared on the day of use and filtered through a 0.2 μm Acrodisc filter (Gelman, Ann Arbor, MI) prior to use. Drug doses are expressed as the base. The possibility that [^{3}H]-METH loss via adsorption onto the filter membrane, thereby lowering the actual amount injected/infused into eggs, was checked. Nine samples were filtered, collected and counted in a scintillation spectrometer (vida infra), which indicated that loss was negligible; recovery was 98.2±2.6% (Mean±SD).

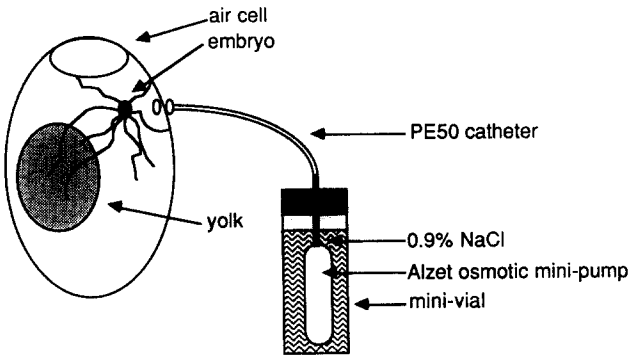

FIG. 1. Externalized Alzet osmotic mini-pump used for continuous infusion of METH near the chicken embryo. Egg and assembly maintained at 37°C in a standard forced-air incubator.

Chronic Methadone Infusion

Catheters were made from PE50 tubing, using a modification of the procedure reported by de Balbian Verster *et al.* [1], in which two bulb-like enlargements were made approximately 1 mm apart. The bulb at the tip, which was to be pressed into the egg, was approximately 1.3 mm in diameter. The second enlargement was slightly larger. The end of the catheter was cut near the smaller bulb and the other end attached to the flow moderator of the mini-pump. Immediately prior to use, the catheter assembly was sterilized by flushing with 70% EtOH, followed by 1.0 ml of sterile saline or the appropriate drug solution. The transparent tape covering the drug delivery hole was removed and the membrane gently pierced with a sterile 25 gauge needle. The smaller bulb of the catheter was inserted into the egg and the tubing was taped to the shell to prevent movement and accidental removal. The osmotic mini-pump was filled according to the manufacturer's instructions, flow moderators inserted and submersed in 3.5 ml of 0.9% NaCl contained in a capped 5.0 ml glass liquid scintillation counting mini-vial (Fig. 1). The egg and osmotic mini-pump assembly were placed in the incubator with the mini-pump assembly in a rack alongside the egg. Eggs were infused at a constant rate of 1 μl/hr for 7 days.

Motility Recording

Two platinum wire (33 gauge) electrodes were used to conduct the electrical potential produced by embryonic movements [4,10]. These electrodes extended 4.0 mm from the end of the barrels of 1.0 ml plastic disposable syringes, which were held in place by micromanipulators, and enclosed in a table top incubator (Forma Scientific Inc., Marietta, OH) maintained at 37°C. Embryonic movements were recorded on a Gilson polygraph recorder, chopper No. ECG-20 (Gilson Medical Electronics, Middleton, WI), which was calibrated daily such that a 0.5 mV internal calibration signal produced a 3.0 cm pen deflection.

To record embryonic motility, the tape covering all holes was removed and the membrane gently pierced with a 25 gauge needle. The egg was placed on a trianglar arrangement of three phonograph cartridges [6], which absorb vibrations, and the electrodes inserted approximately 2 mm into the egg. The embryo was allowed to acclimate for 2 min, at which time 5 min of baseline motility was recorded. Recording continued while Nx was infused (1 mg/kg egg/min at a rate of 2.5 μl/min) for 10 min. In one experiment recording was continued for an additional 5 min after discontinuing acute Nx infusion.

Motility Analysis

Motility recordings were summarized by counting the number of 2 sec intervals (epochs) in which the pen made at least one deflection greater than 1.2 cm above the baseline record of a nonviable embryo [8]. The average number of epochs per min were compiled and used for data analysis.

Extraction Procedure for [^{3}H]-METH

The procedure reported by Misra and co-workers [12] was used, but scaled down to 0.4 volume. Tissue was homogenized 1:4 (w/v) in 0.5 N HCl. For determination of the amount of unchanged [^{3}H]-METH, 0.4 ml unlabeled METH (1.0 mg/ml) was added to an 0.8 ml aliquot of homogenate and the pH adjusted to 9.0 using a predetermined amount of 1.0 N NaOH. After the addition of 1.6 ml of 40% (w/v) K_2HPO_4 and 6.0 ml of ethylene dichloride containing 20% (v/v) isopropanol, the reaction vessel was shaken and centrifuged. The aqueous layer was aspirated and discarded. The organic layer was washed by the addition of 1.6 ml of 4% (w/v) K_2HPO_4. The reaction vessel was shaken, centrifuged and the aqueous layer aspirated and discarded. A 2.0 ml aliquot of the organic layer was removed and evaporated in a glass mini-vial under a fume hood. The residue was dissolved in isopropanol and 2.5 ml Aquasol-2 (NEN Research Products, Boston, MA) used as the scintillation cocktail. To determine total METH, including metabolites, an aliquot of homogenate was added to 1 ml of NCS tissue solubilizer (Amersham, Arlington Heights, IL) and slowly shaken overnight in a 45°C water bath. After cooling, 15 ml of Aquasol-2 was added. All samples were dark adapted for 24 hr and counted for 100 min or 2% error. Appropriate quench curves were used to correct for counting efficiency. No attempt was made to determine isomeric species of METH or specific metabolites in any of the experiments.

Statistics

All motility data was analyzed by linear regression, paired *t*-test and/or ANOVA with repeated measures, followed by an appropriate post hoc test (e.g., Dunnett's using the Satterthwaite corrected MS_{error} term). Body weights were analyzed by one-factor ANOVA, followed by Dunnett's. A Chi-square test was used on the viability data. Chemical data was analyzed using a Student's *t*-test [17]. Data from uninjected and vehicle-injected control groups were combined, when not statistically different.

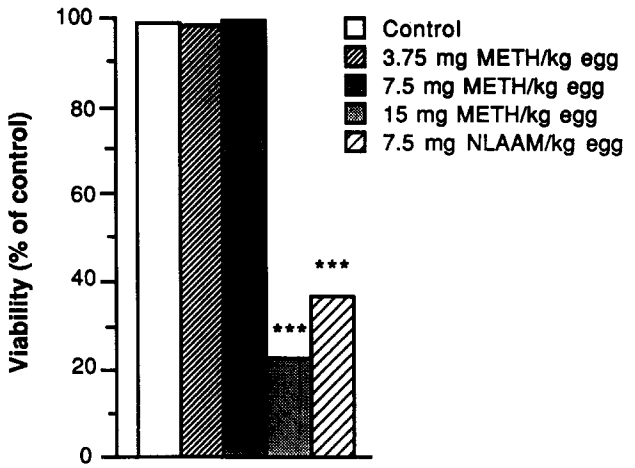

FIG. 2. Viability of 17-day-old embryos injected with METH or NLAAM on day 7 of development. The highest dose of METH (15 mg/kg egg) and NLAAM, (7.5 mg/kg egg), injected on day 7, reduced embryo viability to below 50% of vehicle-injected controls. Injection of vehicle (50% PG) did not have an effect upon viability. ***$p<0.001$, χ^2, N=30–85.

Single Injection of METH

Experiment 1. In an attempt to replicate the NLAAM experiments using METH, we injected [^{3}H]-METH (3.75–15 mg/kg egg), NLAAM (7.5 mg/kg egg) or vehicle (30 μl 50% PG) near 7-day-old embryos. Embryonic motility was recorded on day 17 of development. Following the completion of motility recording, the embyro was quickly removed from the egg, rinsed, blotted dry and weighed. After decapitation, the whole brain and liver were dissected out and weighed. For METH extraction, brains from the embryos exposed to the highest dose of [^{3}H]-METH were frozen on dry ice and stored at −70°C until analysis.

Results. Figure 2 represents the effect of various doses of METH or the dose of NLAAM upon viability on day 17 of embryogenesis. An overall Chi-square analysis, using combined control group's values as 100% of the expected viability (PG group not different from uninjected control), indicated a significant effect of treatment ($\chi^2=108.12$, $p<0.001$). Subsequent analysis confirmed the significant reduction in viability on day 17 for the highest dose of METH (i.e., 20% of control) and the dose of NLAAM (i.e., 37% of control). Of those surviving, only the high dose METH embryos were significantly reduced in body weight (Fig. 3), $F(4,66)=7.1$, $p<0.05$, although liver and brain weights remained unchanged, as a percent of body weight, regardless of group. Even though there was no difference in baseline motilities of the NLAAM and METH groups, compared to controls (Fig. 4), only the embryos exposed to NLAAM showed evidence of dependence. This was indicated by a significant increase in motility above that of control embryos, after acute infusion of Nx. At this time (day 17), small concentrations of METH and metabolites were found in brain; 0.01%, or less,

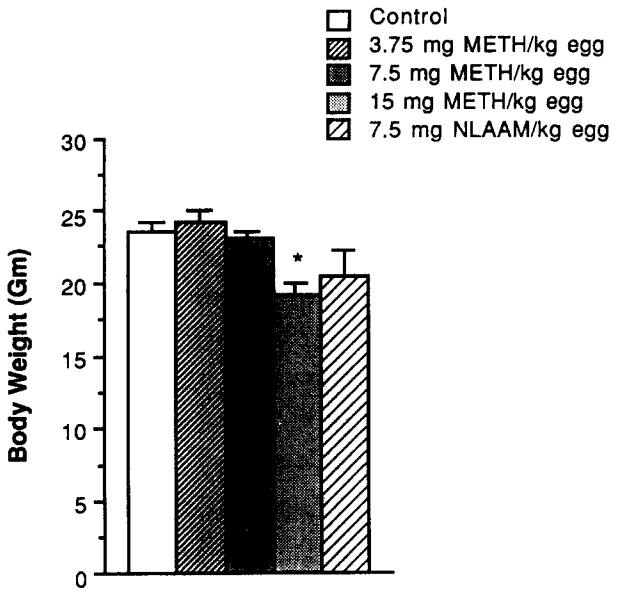

FIG. 3. Body weight (Mean+SE) of 17-day-old embryos injected with METH or NLAAM on day 7 of embryogenesis. Only METH (15 mg/kg egg) produced a significant decrease in body weight. $^*p<0.05$, Dunnett's test, after one-factor ANOVA, N=4–21.

of the injected dose was unchanged METH. There were no differences for any other variables between the uninjected and vehicle-injected groups.

Experiment 2. In the previous experiment the possibility existed that METH produced dependence and subsequent spontaneous withdrawal between the time of injection and time of testing 10 days later. Therefore, we injected METH (5.3 or 10.6 mg/kg egg), NLAAM (5.3 mg/kg egg) or vehicle (30 μl 50% PG) near embryos on day 7 or 11 of development. Embryonic motility was recorded on day 14 or 17. This allowed us to test for evidence of dependence between 3 and 10 days after injection, instead of the customary 10–14 days we had previously used for NLAAM. Following completion of motility recording, embryos were removed from the eggs and weighed. Brains were then dissected out and weighed.

Results. Even as short as 3 days after injection of either opioid, there was no treatment effect on motility, before or after acute infusion of Nx on day 14 of development. Motility of 17-day-old embryos, previously treated with METH either on day 7 or 11, was not depressed and an acute infusion of Nx on this day did not increase motility above Nx infused controls, indicating that they were not experiencing withdrawal, and by inference, not dependent at this time. However, the motility of the NLAAM group injected on either day 7 or 11 was significantly, $F(6,64)=4.7$, $p<0.05$, elevated above vehicle controls in response to acute Nx infusion, indicating opioid withdrawal in ova (Fig. 5). There were no significant differences in brain or body weights between groups.

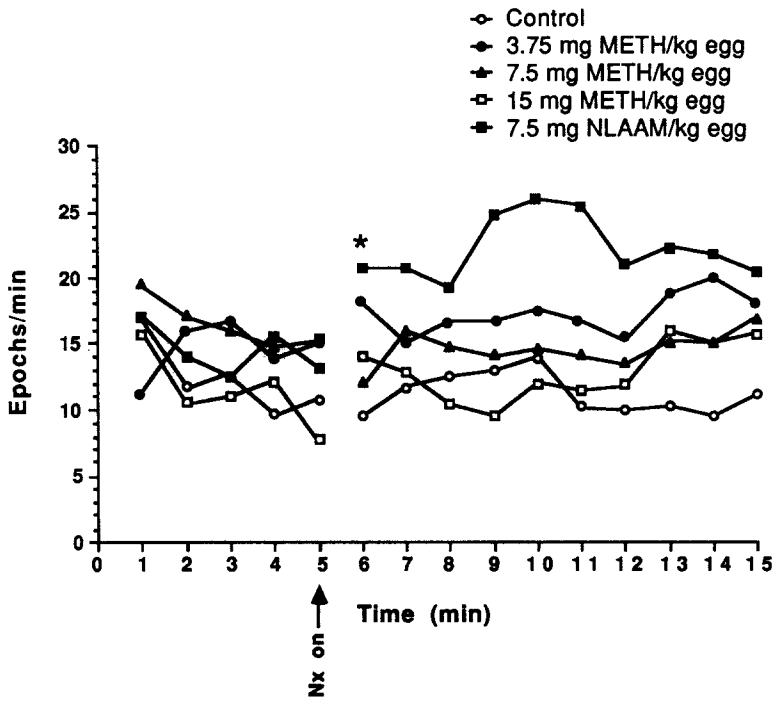

FIG. 4. Motility of 17-day-old embryos, injected with METH or NLAAM on day 7 of embryogenesis, before and after an acute infusion of Nx. Infusion of Nx (10 mg/kg egg/10 min) produced a significant increase in motility only in embryos previously exposed to a single injection of NLAAM (7.5 mg/kg egg). Baseline motilities of embryos previously exposed to either opioid were not different from control. Furthermore, none of the doses of METH were able to sustain opioid-type dependence when measured 10 days after injection. $^*p<0.05$, Dunnett's test, after one-factor ANOVA with repeated measures, N=5–9.

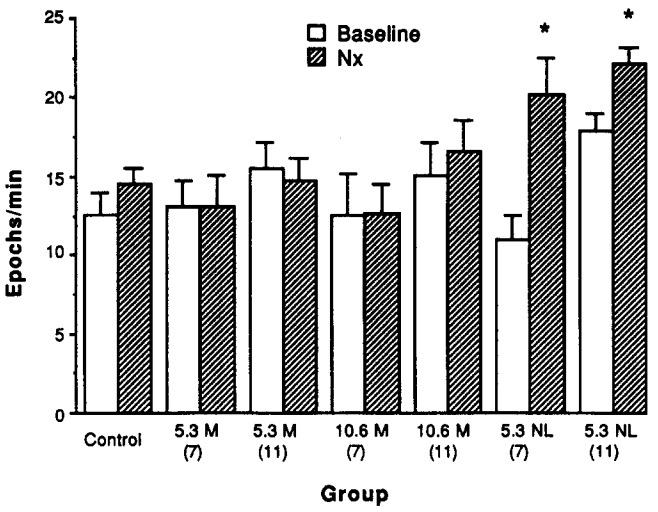

FIG. 5. Motility before and after an acute infusion of Nx in 17-day-old embryos injected with METH or NLAAM on either day 7 or 11 of embryogenesis. Injection of NLAAM (5.3 mg/kg egg) on day 7 or day 11 of embryogenesis resulted in evidence of opioid-type dependence, measured as an increase in motility above contol, after an acute infusion of Nx. This increase was not seen in the eggs injected with METH. *$p<0.05$, Dunnett's test, after one-factor ANOVA with repeated measures. Histograms represent Mean+SE, N=8–18.

Brain Uptake and Elimination of METH After a Single Injection Near 14-Day-Old Embryos

Experiment 3. Because it was apparent that a single METH injection could not sustain evidence of dependence as early as 3 days after injection, this experiment was performed to determine whether a significant amount of METH was getting to the target organ (brain), and to characterize its disposition.

[^{3}H]-METH (30 mg/kg egg in 20 μl water) was injected near 14-day-old embryos using a Tridak Stepper repetitive pipette (Indicon Inc., Brookfield, CT). At predetermined times after injection, eggs were removed from the incubator and embryonic brains rapidly dissected out after decapitation. Brain tissue was frozen on dry ice and stored at −70°C until analysis.

Results. Figure 6 demonstrates that rapid distribution of [^{3}H]-METH to the embryonic brain occurs after a single injection near the embryonic vitelline vessels. Within 5 min the amount of METH in the brain was approximately 20% of its peak concentration. The rapid uptake of METH in this species is similar to that which occurs in the rat [12,14]. The peak concentration of METH, representing approximately 3.4% of the injected dose, occurred 1 hr after injection, at which time no significant amounts of metabolites were found. The apparent $t^1/_2$ for unchanged METH in brain was found to be about 2.8 hr and as expected, the concentration of unchanged METH at 9 hr, 7.7 μg/g, represents approximately 15% of that achieved at 1 hr. Furthermore, at 9 hr, 33% of the radiolabel was metabolized METH, indicating that the 14-day-old embryo is capable of metabolizing the opioid to a great extent.

Chronic Infusion of METH Near 7-Day-Old Embryos

Experiment 4. The results from the preceding experiments demonstrate that multiple daily injections of METH may be required as a method of inducing dependence/withdrawal similar to that seen after a single injection of

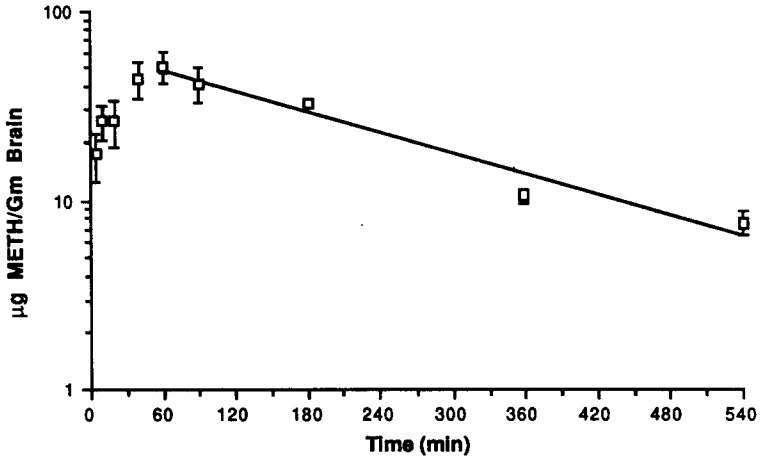

FIG. 6. Uptake of [^{3}H]-METH in 14-day-old embryonic brains. Significant uptake of METH into embryonic brain occurred within 5 min after an injection (30 mg/kg egg) near the embryo. The maximum concentration (Mean±SE) occurred 1 hr after injection, which represents 1.3% of the dose. Brain half-life of the unmetabolized METH was approximately 2.8 hr and at 9 hr 33% of the radiolabel (not shown) was associated with metabolized species of drug. N=8 at each time point.

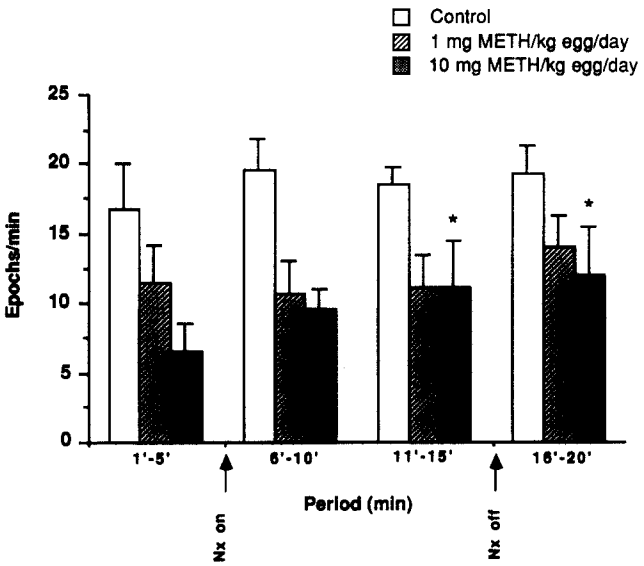

FIG. 7. Motility of embryos infused from day 7 with either METH or saline and tested for opioid dependence with an acute infusion of Nx on day 14 of development. Both doses of METH depressed baseline motility. Infusion of Nx, beginning at 6 min and ending at 15 min, reversed the suppressed motility only in the high dose embryos but they did not attain control levels. Nx did not have an effect on the motility of either the saline or low dose METH embryos. *$p<0.05$, Dunnett's test, after one-factor ANOVA. **$p<0.05$, paired t-test. Histograms represent Mean+SE, N=5.

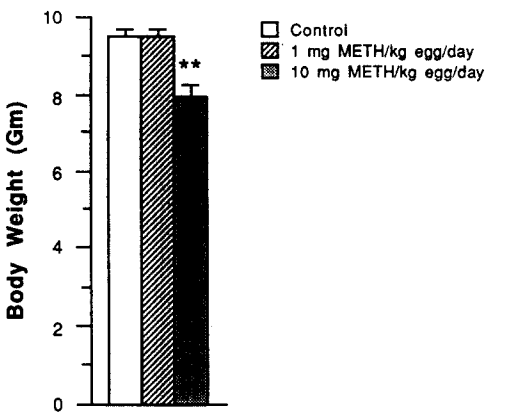

FIG. 8. Body weight (Mean+SE) of 14-day-old embryos after 7 days of continuous exposure to METH. The highest dose of METH (10 mg/kg egg/day) produced a significant reduction in embryonic body weight compared to saline infused eggs. **$p<0.01$, Dunnett's, after one-factor ANOVA. Histograms represent Mean+SE, N=5–9.

NLAAM near the chicken embryo. Because multiple injections may be impractical (vida supra), we used a continuous infusion procedure. [^{3}H]-METH, at doses of either 1 or 10 mg/kg egg/day, or saline, were infused near 7-day-old embryos for 7 days. The amount of radioactivity infused was 1.8 μCi/egg. To maintain isotonicity, saline or water were used as the solvents for the low and high dose groups, respectively.

Motility was recorded on day 14, as above, with an additional 5 min of recording after terminating the acute Nx infusion. After the completion of the motility recording, embryos were weighed and the embryonic brains were quickly dissected. Brain tissue was frozen on dry ice and stored at −70°C until analysis.

Results. Figure 7 shows that chronic infusion of METH, from day 7 to day 14 of development, caused a significant depression of embryonic baseline motility [linear, $F(1,13)=7.22$, $p<0.05$]. Only the motility of the embryos exposed to the high dose of METH were significantly reduced compared to controls [$F(2,12)=3.34$, $p<0.05$]. Acute infusion of Nx failed to increase motility of either METH group above that of the control group. Although the motility of the embryos exposed to the high dose of METH remained significantly suppressed, relative to controls, the acute infusion of Nx was capable of significantly increasing the suppressed motility above baseline values ($p<0.05$, paired t-test).

Analysis of embryonic body weights revealed a significant difference due to treatment [$F(2,19)=11.7$, $p<0.001$]. Subsequent contrasts revealed that the difference was due to a significant reduction in body weight in the high dose group. The low dose of METH did not affect this variable (Fig. 8). Brain weights were unaffected by either dose of infused METH.

The amount of unchanged METH per gram of brain tissue is shown in Table 1. It is also expressed as the percent of the total amount of METH infused during the 7-day period. Interestingly, although the high dose group received 10 times more METH than the low dose group, brain concentrations in the former group were 30 times higher ($p<0.005$, Student's t-test).

TABLE 1

CONCENTRATION AND PERCENT OF INFUSED METH THAT IS UNCHANGED IN THE 14-DAY-OLD EMBRYONIC BRAIN AFTER CHRONIC INFUSION OF METH BEGINNING ON DAY 7 OF EMBRYOGENESIS

Treatment	μg/g Brain	% of infused Dose
1 mg METH/kg egg/day	0.42* ±0.04	0.039% ±0.004%
10 mg METH/kg egg/day	14.13† ±3.10	0.128%† ±0.023%

*Mean (±SE) of METH which is unchanged.
†$p<0.005$ by Student's t-test, N=8 or 9.

DISCUSSION

We attempted to produce somewhat equivalent degrees of embryotoxicity with higher doses of METH and NLAAM so as to ensure exposure, during development, to sufficient drug concentrations which would maximize our chances of inducing dependence. Even though METH (15 mg/kg egg) injected on day 7 reduced viability to about 20% of control and also reduced body weight of the survivors on day 17, none of the doses of METH (i.e., 3.75–15 mg/kg egg) were able to sustain opioid-type dependence. On the other hand, 7.5 mg NLAAM/kg egg, as expected, was able to do so. Only the NLAAM group responded with a significant increase in motility, after acute infusion with Nx on day 17.

The decrease in spontaneous motility observed on day 19 by Kuwahara and Sparber [8] but not observed in this study on day 17 of development in embryos exposed to higher doses of NLAAM (7.5 mg/kg egg) is probably due to the

beginning of the hatching process in the older embryos. Hatching involves resorption of the yolk and other remaining sources of nourishment used by the neonate just after hatching, which may be responsible for the surge in NLAAM concentration in the chick brain at this time [7]. Thus, on day 17, resorption would not have begun and this could account for the lack of depressed motility at that time of embryogenesis. Because only trace concentrations of unchanged METH (e.g., 0.01%, or less, of the injected dose) were present in embryonic brain 10 days after injection (i.e., on day 17), the inability of a single injection of METH to produce dependence is probably due to the fact that METH is metabolized to an inactive compound(s), while NLAAM is metabolized to an active compound [13].

Because we observed what appeared to be a large peak concentration of METH shortly after injection, and a relatively short half-life in the 14-day-old chicken embryo, it is clear that multiple injections would be necessary, but still may not be sufficient, to produce opioid-type dependence without periods of acute toxicity, followed by periods of spontaneous withdrawal, unless METH was injected at least once daily. By using an externalized Alza osmotic minipump, the problems associated with multiple dosing schedules can be overcome by continuous infusion of drugs (e.g., METH) which are acutely toxic when injected as a bolus, but nevertheless may be rapidly metabolized by the immature organism. Chronic infusion of either a low or a high dose of METH (1 or 10 mg/kg egg/day) for 7 days (day 7–14 of development) resulted in significant dose-dependent amounts of unchanged METH in the brain on day 14 of development. The higher than expected concentration of unchanged METH in the brains of embryos exposed to the high dose, relative to the low dose, indicates general developmental retardation (i.e., lower drug metabolism capacity), saturation of the mixed function oxidase system and/or a direct toxic action upon drug metabolizing organs. Furthermore, it is possible that embryos exposed to the lower dose of METH may have induced the METH metabolizing enzymes. In support of the former possibilities, Jakubovic *et al.* [5] found that daily injections of 0.08 and 0.41 mg METH/kg egg/day into the air sac from day 2 of development produced a significant decrease in brain protein concentration, measured on day 13, and a decrease in liver weight in the higher dose group, without a significant decrease in viability. No attempt was made to determine if chronically exposed embryos were opioid-dependent or would eventually hatch.

Our inability to demonstrate an overshoot, or significant increase in motility above controls, in either the NLAAM- or METH-exposed embryos on day 14, after Nx injection, suggests that the "machinery" (biochemical, physiological or structural) necessary for the full adaptive response (dependence) or its expression (withdrawal), as measured, may not yet be developed. Since opioid receptors have been reported to appear as early as day 5 in chicken embryos [2,11], and are similar to those in the adult, the differential effects of METH and NLAAM (i.e., being able to show dependence on day 17, after NLAAM) may be due to the greater affinity of NLAAM over METH for opioid receptors in embryonic chick brain [3]. Additional research will be necessary to determine why dependence was not demonstrable on day 14 after exposure to METH or NLAAM. For example, it is possible that a moderate degree of dependence did exist after 7 days of chronic infusion of the low dose of METH. Since the older embryo is able to metabolize and/or redistribute METH out of the brain readily, the low dose was too low and the high toxic dose could not be completely antagonized by the high dose of Nx injected. Perhaps a higher acute dose of Nx might have unmasked dependence in either group.

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