Prenatal Opiate Withdrawal Activates the Chick Embryo Hypothalamic-Pituitary-Adrenal Axis and Dilates Vitelline Blood Vessels via Serotonin$_2$ Receptors

LISA M. SCHROTT, MARY IRENE BAUMGART, XUEWEI ZHANG, and SHELDON B. SPARBER

Department of Pharmacology (L.M.S., M.I.B., X.Z., S.B.S.) and Graduate Program in Neuroscience (S.B.S.), University of Minnesota, Minneapolis, Minnesota

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ABSTRACT

Opiate withdrawal during pregnancy may occur because of voluntary or forced detoxification, or from rapid cycling associated with exposure to short-acting “street” opiates. Thus, animal modeling of prenatal withdrawal and development of potential therapeutic interventions is important. Direct developmental effects of opiates and/or withdrawal can be studied using a chick model. In ovo administration of the long-acting opiate N-desmethyl-l-$\alpha$-noracetylmethadol (NLAAM) induces opiate dependence in the chick embryo. We have examined activation of the hypothalamic-pituitary-adrenal (HPA) axis (assessed via serum corticosterone) and hemodynamic changes (assessed as changes in apparent diameter of vitelline (extraembryonic) blood vessels) after chronic NLAAM exposure and naloxone (Nx)-precipitated withdrawal during late stages of embryogenesis. Nx-precipitated withdrawal increased corticosterone 2–4.5-fold and diameters of vitelline blood vessels by 15 to 45%. NLAAM exposure itself did not effect these measures. In a second set of experiments, isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor, was injected into eggs with embryos. IBMX similarly increased corticosterone and vitelline vessel diameter, with a similar time course and response magnitude. Previous studies found that serotonin$_2$ (5-HT$_2$) receptors were involved in other withdrawal manifestations, so we determined whether they were likewise involved. Pretreatment with the 5-HT$_2$ antagonist ritanserin completely blocked HPA axis activation and vasodilation associated with both Nx-precipitated withdrawal and IBMX administration. This indicates that 5-HT$_2$ receptors, directly or indirectly, mediate these withdrawal manifestations in the chick embryo.

Heroin use during pregnancy is associated with medical complications and current guidelines recommend maintenance on substitution therapy with methadone (Mitchell, 1995). Detoxification is not recommended during pregnancy because of potential fetal distress. Studies in humans have found changes in fetal gross activity and fetal excess meconium, and altered catecholamine concentrations in amniotic fluid during withdrawal (Zuspan et al., 1975; reviewed by Kandall et al., 1999). However, voluntary or forced detoxification during pregnancy may occur and thus, animal modeling of prenatal withdrawal and development of potential therapeutic interventions is important.

Although it has been well characterized in the adult, the manifestations of opiate withdrawal in the fetus are less established. Opiate withdrawal in fetal sheep activates the sympathetic nervous system and alters blood pressure, blood oxygen content, and causes meconium staining (Cohen et al., 1980; Umans and Szeto, 1985). Increased motoric activity after prenatal opiate withdrawal has also been reported in externalized rodent fetuses (Kirby and Holtzman, 1982; Jones and Barr, 2000). However, it is unclear whether other aspects of opiate withdrawal documented in the adult, such as activation of the hypothalamic-pituitary-adrenal (HPA) axis (for review, see Pechnick, 1993) occur in the immature subject as well.

Using an avian model system, direct effects of opiates and opiate withdrawal, as well as potential remediation strategies, can be studied. We have established procedures to reliably induce opiate dependence in chick embryos using the long-acting opiate N-desmethyl-l-$\alpha$-noracetylmethadol (NLAAM).

ABBREVIATIONS: HPA, hypothalamic-pituitary-adrenal; NLAAM, N-desmethyl-l-$\alpha$-noracetylmethadol; Nx, naloxone; E, embryonic day; IBMX, isobutylmethylxanthine; 5-HT, 5-hydroxytryptamine (serotonin); ANOVA, analysis of variance; PLSD, protected least significant difference.
NLAAM is an active metabolite of l-α-acetylmethadol, an opiate used in substitution therapy for treatment of heroin addiction (Trueblood et al., 1978). NLAAM is administered in ovo by injection underneath the shell into the allantoic fluid and is distributed to the embryo via transport through extraembryonic vasculature.

The present study examined HPA axis activation and hemodynamic changes after chronic opiate exposure and naloxone (Nx)-precipitated withdrawal, as assessed via serum corticosterone and changes in apparent diameter of vitelline (extraembryonic) blood vessels, respectively. The vitelline vessels are analogous to mammalian placental vasculature and regulate nutrient and gas flow. We studied withdrawal at embryonic day (E) 15 and E18 (out of a 21-day incubation period). At these ages the chick HPA axis is functional (Bordon et al., 1997) and sensitive to other drug-induced changes (Larson et al., 2001). Alterations in blood vessel diameter also cause pathology at these embryonic ages (Sparber et al., 1996; Zhang et al., 1998).

The consequences of prenatal opiate exposure may result from the direct effects of the opiate on the developing organism and/or the withdrawal manifestations. There has been much interest in the potential mechanisms for the receptor and signal transduction adaptations and compensatory responses during opiate dependence. Potential pathways include changes in cAMP-mediated responses, mobilization of intracellular Ca\(^{2+}\), altered protein kinase signaling, and changes in K\(^+\) and L-type Ca\(^{2+}\) channel gating (for review, see Christie et al., 1997). To determine whether cAMP-mediated changes were a possible mechanism for the effects of opiate withdrawal in the chick embryo, we administered 3-isobutyl-1-methylxanthine (IBMX) to eggs containing chicken embryos. IBMX is a phosphodiesterase inhibitor that increases intracellular cAMP. Compared with other xanthines, IBMX has relative selectivity for cAMP phosphodiesterase and this may be a key factor for its usefulness in understanding the signaling associated with opiate withdrawal (Butt et al., 1979). Previous studies showed that IBMX mimics many of the behavioral and physiological changes seen in withdrawing opiate-dependent chicks and rodents (Collier et al., 1981; Grant and Redmond, 1982; Bronson and Sparber, 1989; Kleven and Sparber, 1989a,b). IBMX also exacerbates the effects of precipitated opiate withdrawal (Holtzman, 1989). The effects of IBMX can be blocked by opiate administration in a dose-dependent manner (e.g., Collier et al., 1981; Kleven and Sparber, 1989a) further suggesting similar downstream pathways between opiate withdrawal and IBMX. Thus, we administered IBMX to chick embryos and examined HPA axis activation and blood vessel diameter.

Serotonin\(_2\) receptors have also been implicated in the consequences of both opiate withdrawal and IBMX administration. Somatic, behavioral, and physiological indices of both opiate withdrawal and IBMX administration can be blocked or attenuated by pretreatment with a variety of 5-HT\(_2\) receptor antagonists (Neal and Sparber, 1986; Kleven and Sparber, 1989b). We thus determined whether changes in HPA axis and blood vessel diameter caused by Nx-precipitated withdrawal or IBMX administration were ameliorated by ritanserin pretreatment.

Materials and Methods

Subjects

Fertilized White Leghorn eggs were obtained from the Poultry Nutrition Research Center (University of Minnesota, St. Paul, MN). The eggs were set in a rotating forced air incubator/hatcher (Humidaire, New Madison, OH) with the temperature at 37–38°C and the relative humidity at 58 to 60%. The day the eggs were set was designated as E0. To locate an injection site for drug administration the eggs were candled and a site that avoided membrane-bound blood vessels was marked about 2.0 cm below the air cell. The shell surface at the injection site was cleaned and a 1.2-mm-diameter dental burr, attached to a small drill was used to make injection holes. Care was taken not to puncture the underlying membrane and holes were covered with a small piece of transparent plastic tape. Appropriate drug or vehicle solutions were administered via injection 2 to 3 mm beneath the shell of the egg. For more details, see Schrott et al. (1999).

Treatments

Experiment 1. NLAAM (kindly provided by the National Institute on Drug Abuse) or its vehicle, 50% propylene glycol, was injected into eggs containing E4 embryos. The doses of NLAAM ranged from 2.5 to 10 mg/kg of egg weight (approximately 0.14–0.60 mg/egg). These doses are known to reliably induce chronic opiate dependence that lasts throughout the 21-day incubation period (Kuwahara and Sparber, 1981). To precipitate withdrawal on E15 or E18 naloxone HCl (Nx; Sigma-Aldrich, St. Louis, MO) was administered at a dose of 10 mg/kg egg weight. Control subjects received an avian saline injection (0.85% NaCl). Drug solutions were made fresh and the injection volume was 20 μl/egg.

Experiments 2 and 3. NLAAM (5 mg/kg) or vehicle was administered on E3 or E4 as described for experiment 1. On E14 and E17, approximately 16 to 24 h before precipitation of withdrawal, the 5-HT\(_2\) antagonist ritanserin HCl (Sigma/RBI, Natick, MA; now distributed by Sigma-Aldrich) or its vehicle, 0.1 M tartaric acid, was injected into eggs containing embryos. The ritanserin dose was 0.9 mg/kg of egg weight, a dose that has no toxicity at these ages (Bollweg et al., 1998; Schrott et al., 1999) but is effective in blocking excess 5-HT\(_2\) activation in the chick embryo (Sparber et al., 1996). Withdrawal was precipitated with Nx on E18 as in experiment 1. For the blood vessel experiment on E15, Nx was infused over a 10-min period at a rate of 0.5 mg/kg egg/min (total infusion volume 50 μl). All other injection volumes were 20 μl.

Experiments 4 and 5. For the initial study, IBMX (Sigma-Aldrich), its vehicle 5% pluronic F68 polyol (BASF Wyandotte Corp., Wynadotte, MI), or avian saline was administered on E18. The IBMX dose was 10 mg/kg of egg weight and was delivered in a volume of 80 μl. For the second IBMX study, ritanserin was administered on E17 as described for experiments 2 and 3, followed by IBMX or its vehicle on E18.

Procedures

Blood Collection. Blood samples were obtained from the chick embryos via cardiac puncture 30 min after precipitation of opiate withdrawal in experiments 1 and 2. For the initial IBMX study in experiment 3, blood samples were taken at 30 min, 1 h, or 2 h after IBMX. Blood samples were obtained within 2 min of removing the embryo from the incubator. Sera were isolated from the blood and stored at −70°C until analyzed for corticosterone via radioimmunoassay.

Radioimmunoassay for Serum Corticosterone. The sera were diluted 1:50 and heated at 100°C for 10 min to denature corticosterone binding globulin. The samples were then incubated with an antibody directed against corticosterone (ICN Pharmaceuticals, Costa Mesa, CA) and \(^{3}H\)corticosterone (PerkinElmer Life Sciences, Boston MA). Charcoal was added and samples were
centrifuged to separate bound \([^{3}H]\)cORTicosterone. Bound \([^{3}H]\)cORTicosterone was counted in a liquid scintillation counter to an error of \(\pm 1.5\%\). All samples and standards were run in triplicate. Standards were used to generate a curve from which sample values were interpolated. These values were subsequently converted and expressed as nanograms per milliliter of serum. Assay sensitivity was between 0.5 and 1 ng/ml, assay range from 1 to 1000 ng/ml, and the intra-assay coefficient of variation was approximately 5%.

**Measurement of Blood Vessel Diameter.** A circular 3- to 4-cm-diameter hole was made over the aircell using a special puncturing device. The partial eggshell was carefully removed and mineral oil was applied to the exposed membrane to make it transparent. The egg was placed upright in an incubator maintained at 37°C on a sponge cradle under an endoscope connected to a Toshiba color camera-Panasonic digital mixer-VCR system. The endoscope was lowered until a vitelline blood vessel was in focus when viewed on a television monitor. A 1-cm, 250-µm-diameter silk suture that had been soaked in mineral oil was placed near the desired blood vessel to serve as a reference. After a 5-min acclimation period, a baseline video image of the selected blood vessel and suture were recorded. Injection or infusion of Nx or its vehicle followed the baseline recording. At 5, 10, and 15 min postinjection (or infusion) additional video images of the blood vessels were recorded. For more details on this procedure, see Zhang et al. (1998). For data analyses, frames were taken from the recordings and the diameter of the reference suture and the selected blood vessel were measured using NIH Image software (National Institutes of Health, Bethesda, MD). For the experiment conducted on E15, a single, approximately 200-µm-diameter blood vessel was chosen. For the experiments on E18, two blood vessels were assessed, one large (approximately 300 µm in diameter) and one small (approximately 100 µm in diameter), to determine whether effects were influenced by vessel size. Measurements, in pixels, were converted to micrometers by comparison using the suture diameter as a reference. Because of the large variability between subjects, the values are expressed as a percentage of the individual baseline values. The experimenter analyzing the images was blind as to treatment.

**Statistical Analyses.**

In the present studies, multiple drug treatments were administered to the embryos. For the corticosterone analyses, a control group that received opiate exposure without undergoing withdrawal (E4 NLAAM + E15 or E18 vehicle) and a group receiving Nx in the absence of opiate withdrawal (E4 vehicle + E15 or E18 Nx) was included such that nonselective drug effects could be ruled out. For the blood vessel diameter studies, baseline measures were taken before Nx administration that allowed us to rule out nonselective effects in the absence of separate control groups. Initial analyses were done to determine whether NLAAM or Nx on their own affected these measures.

For the NLAAM-Nx corticosterone experiments (experiments 1 and 2), the data were analyzed by one-way analysis of variance (ANOVA). For the IBMX study that examined corticosterone concentrations across time (experiment 4), two-way ANOVA with Treatment and Time as the between-subjects measures was used. Changes in apparent blood vessel diameter were analyzed with a repeated measures ANOVA, with time postinjection as the within-subjects factor and treatment as the between-subjects factor (experiments 3 and 5). Planned contrasts between treated and control subjects were made using Dunnett’s tests, or Fisher’s PLSD tests, for effects between treated groups when appropriate. Because the direction of the effects (increased corticosterone during withdrawal) was predicted for experiments 1, 2, and 4, unidirectional contrasts were used.

### Results

**Experiment 1: Does Naloxone-Precipitated Opiate Withdrawal Increase Serum Corticosterone on E18 or E15?**

**E18 Corticosterone.** The first study was conducted on E18 because we had evidence from previous studies that the E18 chick embryo HPA axis is capable of mounting a corticosterone response to a pharmacological challenge (Larson et al., 2001). Three doses of NLAAM (2.5, 5, or 10 mg/kg egg) or vehicle were administered on E4. On E18 Nx was injected into the eggs to precipitate withdrawal or saline was injected to determine whether corticosterone concentrations were affected by chronic NLAAM exposure. There were six to 10 subjects per treatment group. There was no effect of chronic NLAAM exposure itself on corticosterone concentrations for subjects exposed to saline on E18 (mean ± S.E.M.: E4 vehicle = 4.03 ± 0.47 ng/ml; 2.5 NLAAM = 4.44 ± 1.05 ng/ml; 5 NLAAM = 4.42 ± 1.08 ng/ml; and 10 NLAAM = 3.49 ± 0.50 ng/ml). There was also no effect of acute E18 Nx on embryos from eggs treated with vehicle on E4 (mean ± S.E.M.: E4 vehicle + E18 saline = 4.03 ± 0.47 ng/ml versus E4 vehicle + E18 Nx = 5.04 ± 0.96 ng/ml). Therefore, these two groups were combined to make a control group for subsequent analyses.

An overall Treatment effect was found when comparing the three E4 NLAAM + E18 Nx groups and the combined control groups (\(F_{3,38} = 4.88, p < 0.02\)). NLAAM + Nx increased serum corticosterone, with an approximate 1.7-fold increase at 2.5- and 5-mg NLAAM/kg of egg weight doses and a 3-fold increase at the 10-mg NLAAM/kg dose. The mean ± S.E.M. were as follows: combined control = 4.51 ± 0.51 ng/ml; 2.5 NLAAM + E18 Nx = 7.69 ± 1.26 ng/ml; 5 NLAAM + E18 Nx = 7.62 ± 1.67 ng/ml; and 10 NLAAM + E18 Nx = 11.79 ± 2.66 ng/ml). In other studies, we have found the 10-mg/kg dose of NLAAM to significantly decrease embryo viability by 30%, or more, compared with vehicle-treated controls (L. M. Schrott and S. B. Sparber, unpublished observations). This suggests that alterations in the HPA axis after the 10-mg/kg dose may be secondary to toxicity and that it may be an inappropriate dose to mimic potential methadone or l-α-acetylmethadol exposure in the human fetus. Therefore, we excluded this dose and reran the analysis using the Nx-treated subjects exposed to the lower two doses of NLAAM. The overall treatment effect remained significant (\(F_{2,30} = 3.81, p < 0.04\)). Planned comparisons revealed significant differences between both treated groups and the combined control group (\(p < 0.035\) or better, Fisher’s one-tailed PLSD). Thus, in the remaining studies we used the 5-mg NLAAM/kg dose.

**E15 Corticosterone.** A similar study was conducted on E15 to determine whether younger embryos would also manifest opiate withdrawal with increased circulating corticosterone. Eggs with E4 chick embryos were administered vehicle or 5 mg NLAAM/kg of egg weight, followed by saline or Nx on E15. ANOVA revealed a significant effect of E4 + E15 treatment (\(F_{3,28} = 18.95, p < 0.0001\)). As was seen on E18, E4 NLAAM exposure on its own did not affect serum corticosterone in embryos from eggs administered saline on E15 (mean ± S.E.M.: E4 vehicle = 2.80 ± 0.36 ng/ml; and E4 5 NLAAM = 3.41 ± 0.30 ng/ml). Nx on E15 also did not affect corticosterone in E4 vehicle-treated subjects (mean ± S.E.M.:
Experiment 2: Can Ritanserin Pretreatment Block the Corticosterone Elevation Induced by Naloxone-Precipitated Withdrawal?

Ritanserin (0.9 mg/kg) or its vehicle was administered the day before Nx-induced opiate withdrawal to determine whether blockade of 5-HT₂ receptors could prevent the activation of the HPA axis. In previous studies we had demonstrated that ritanserin administered on E17 had no effect on serum corticosterone on E18 (Larson et al., 2001). Thus, this control group was not included in the study examining effects on E18. However, an E14 ritanserin-only group was included to determine whether there were effects of this treatment on E15 corticosterone.

E18 Corticosterone. Figure 1 (top) shows that ritanserin administered on E17 blocked the corticosterone increase caused by opiate withdrawal on E18 (treatment effect $F_{5,24} = 2.96, p = 0.05$). An approximate 2-fold increase in corticosterone was found in the embryos undergoing opiate withdrawal (NALAM + Nx) that were pretreated on E17 with vehicle, similar to that seen in experiment 1. Ritanserin administration on E17 completely blocked this effect.

E15 Corticosterone. Initial analyses revealed no differences among embryos from eggs treated on E4 with vehicle that was followed by either ritanserin or vehicle on E14 and Nx or vehicle on E15 (mean ± S.E.M. of these groups ranged from $2.05 \pm 0.54$ to $3.01 \pm 0.54$ ng/ml; $n = 7$–9). For the remaining comparisons, the group administered vehicle at E4 and E14 and Nx on E15 was used as the control group. A similar pattern was found on E15 as was seen on E18, with an overall treatment effect ($F_{2,30} = 13.65, p < 0.0001$; Fig. 1, bottom). Increased serum corticosterone was again found in the embryos undergoing opiate withdrawal, with the magnitude of the effect on E15 (3.5-fold increase) a bit greater than that of E18 (2-fold increase). Ritanserin pretreatment blocked this effect, with these embryos having corticosterone concentrations similar to those of the controls.

Experiment 3: Can Ritanserin Pretreatment Block Changes in Blood Vessel Diameter Associated with Naloxone-Precipitated Withdrawal?

E18 Blood Vessel Diameter. We had previously demonstrated that ritanserin on its own does not affect vitelline blood vessel diameters in this age range (Zhang et al., 1998). Thus, for these experiments, the following groups were compared: embryos treated on E4 with vehicle or 5 mg NLAAM/kg, followed by ritanserin or vehicle on E17 and Nx on E18. Initial analyses were done on baseline blood vessel diameters to determine whether NLAAM on its own affected the diameters. E4 NLAAM on its own did not affect the diameters of either large (micrometer diameter ± S.E.M: E4 NLAAM = 283.38 ± 23.64 versus E4 vehicle = 285.84 ± 19.23) or small blood vessels (micrometer diameter ± S.E.M: E4 NLAAM = 96.67 ± 8.16 versus E4 vehicle = 98.29 ± 8.09). Figure 2 displays captured video images of the vitelline blood vessel before and after injections of Nx. Changes in blood vessel diameters were measured at 5, 10, and 15 min after Nx administration to the egg. The data are expressed as the percentage of change from the baseline diameters and the large and the small blood vessels were analyzed separately. For both large and small blood vessels, there was a significant treatment effect ($F_{3,47} = 7.42$ and $14.45, p < 0.0004$ and 0.0001, respectively). For the small blood vessels, there was also a significant time effect ($F_{2,94} = 9.33, p < 0.0002$), with the largest effects found 10 min after Nx injection. There was no time × treatment interaction. Figure 3 (top) displays the mean ± S.E.M. for the various treatment groups for the small blood vessels. Nx administration on E18 to NLAAM-dependent embryos increased blood vessel diameters by 25 to 45%. Ritanserin pretreatment on E17 blocked this effect, with embryos in this treatment group not different from vehicle-treated controls. Similar effects were found for the large blood vessels; opiate withdrawal increased the diameters by 15 to 20% ($p < 0.05$ at each postinjection time versus controls; Fisher’s PLSD) and again ritanserin pretreatment blocked this effect (data not shown).

E15 Blood Vessel Diameter. For the E15 embryos, a single blood vessel was chosen and similar analyses con-
duted. Initial analyses were done on baseline blood vessel diameters to determine whether NLAAM on its own affected this measure. There were no differences between the diameters of E15 vitelline blood vessels in embryos from eggs treated on E3 with NLAAM (186.97 μm ± 22.03) compared with vehicle (191.25 μm ± 14.79). Changes in blood vessel diameters were measured at 5, 10, and 15 min after Nx infusion. The data are expressed as the percentage of change from the baseline diameters. There was a significant treatment effect (F_{5,24} = 7.31, p < 0.002), with the largest effects found 10 to 15 min after Nx infusion. There was no time × treatment interaction. Figure 3 (bottom) displays the mean ± S.E.M. for the various treatment groups. Nx infusion on E15 into eggs with NLAAM-dependent embryos increased blood vessel diameters by approximately 20 to 30%. Ritanserin pretreatment on E14 blocked this effect as well.

**Experiment 4: Does IBMX Increase Serum Corticosterone via 5-HT₂ Receptors?**

Although previous studies demonstrated that IBMX mimicked motoric signs of opiate withdrawal in the chick embryo (Bronson and Sparber, 1989), it was not known whether this extended to the activation of the HPA axis, and if so, whether the timing was similar. Therefore, the initial study determined when acute IBMX exposure activated the HPA axis on E18. The vehicle used for IBMX was 5% pluronic F68. Because we had not worked with the vehicle in studies examining HPA axis effects, we first determined that it did not alter corticosterone. Samples taken at 30 min postinjection revealed no difference between embryos from eggs administered saline (mean ± S.E.M. = 6.20 ng/ml ± 1.78) or 5% pluronic vehicle (mean ± S.E.M. = 6.30 ± 1.53 ng/ml). When the IBMX-treated groups were compared with vehicle-treated groups, there was a significant effect of treatment (F_{1,28} = 8.16, p < 0.009), but no time nor treatment × time interactions. IBMX increased serum corticosterone approximately 2.5-fold compared with vehicle-treated controls at 30 min (mean ± S.E.M.: IBMX = 16.85 ± 4.18 ng/ml versus vehicle = 6.20 ± 1.78 ng/ml) and 1 h postinjection (IBMX = 15.38 ± 3.97 ng/ml versus vehicle = 6.08 ± 1.68 ng/ml). By 2 h postinjection, there was no difference between the IBMX- and vehicle-treated groups (IBMX = 10.25 ± 2.65 ng/ml versus vehicle = 8.75 ± 2.41 ng/ml). Note that both the magnitude and the timing of the corticosterone increase were similar to that seen after Nx-precipitated withdrawal. Thus, for the subsequent study, blood samples were taken 30 min post-IBMX injection.

To determine whether pretreatment with ritanserin blocked the effects of IBMX, ritanserin was administered on E17 and IBMX on E18. There was an overall effect of treatment (F_{3,31} = 3.06, p < 0.05). As was seen with Nx-precipitated withdrawal, ritanserin blocked the significant IBMX-induced increase in corticosterone, with the ritanserin + IBMX-treated subjects not different from vehicle-treated controls (Fig. 4).

**Experiment 5: Does IBMX Alter Vitelline Blood Vessel Diameter via 5-HT₂ Receptors?**

Diameters of small and large blood vessels were measured in embryos treated on E17 with the 5-HT₂ antagonist ritanserin or vehicle followed by IBMX or vehicle on E18. Changes were measured at 5, 10, and 15 min after IBMX and expressed as the percentage of change from their respective baseline diameters. As was done for experiment 3, the large and the small blood vessels were analyzed separately. The baseline diameter for large blood vessels was 289.94 ± 22.53 μm and for small blood vessels was 83.40 ± 4.83 μm. For both large and small blood vessels, there was a significant treatment effect (F_{2,12} = 7.49 and 17.71, p < 0.001 and 0.0005, respectively). For the small blood vessels, there was also a significant treatment × time interaction (F_{4,24} = 3.73, p < 0.02), with the magnitude of the effects increasing from 5 to 15 min. As can be seen in Fig. 5, IBMX led to vasodilation of the small vitelline blood vessels on E18, increasing vessel diameters by 20 to 55%. This effect was of a similar magnitude as was seen after Nx-precipitated withdrawal. Ritan-
serin pretreatment on E17 likewise blocked this effect, with embryos in this treatment group not different from vehicle-treated controls. Similar effects were found for the large blood vessels, with IBMX increasing the diameters by approximately 20 to 35% (p < 0.05 at each postinjection time versus vehicle-treated control; Fisher’s PLSD) and again ritanserin pretreatment blocked this effect (data not shown).

Discussion

Antagonist-precipitated opiate withdrawal activated the HPA axis and increased diameters of extraembryonic blood vessels at late stages of embryogenesis. Withdrawal, rather than chronic opiate exposure, caused these effects because chronic NLAAM itself did not affect these measures. IBMX mimicked the effects of Nx-precipitated withdrawal, suggesting that similar downstream signaling pathways are involved in these processes. Pretreatment with the 5-HT2 antagonist ritanserin completely blocked the HPA axis activation and vasodilation associated with both Nx-precipitated withdrawal and IBMX administration. The data indicate that 5-HT2 receptors directly or indirectly mediate these withdrawal effects in the chick embryo, similar to that observed previously in developing and mature rats (i.e., Neal and Sparber, 1986; Kleven and Sparber, 1989b, 1991).

Opiate Withdrawal and HPA Axis. The present findings indicate that opiate withdrawal, in addition to other pharmacological effects, can be considered a general stressor to the developing organism. These studies examined direct drug effects on the embryo, rather than indirect responses to changes in the mother that occur during withdrawal. Thus, even if the mother does not mount a detectable HPA axis response to withdrawal, the data suggest that a fetus, during late development, may experience stress. Interestingly, chronic exposure to NLAAM on its own did not affect corticosterone. Because NLAAM was administered on E4 and blood sampling occurred on E15 or E18, this may have been a consequence of opiate tolerance, as has been seen in neonatal rats (Little and Kuhn, 1995). There are reports that chronic opiate treatment can activate the rodent HPA axis...
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(For review, see Pechnick, 1993). However, such studies generally used high doses of opiates (doses well beyond the analgesic dose) and effects may have been due to dysphoria or secondary to toxic effects, such as respiratory depression. Additionally, effects may have been confounded by withdrawal, depending on when blood sampling occurred (for a comprehensive review, see Pechnick, 1993). In the present study, HPA activation after opiate withdrawal was found on both E15 and E18. At E15, the chick HPA axis is functional with respect to increased corticosterone after a “stressor”, but negative feedback pathways have not been established. However, by E18 there is negative feedback regulation of the HPA axis (Bordone et al., 1997). The magnitude of the effect was greater at E15 than E18, suggesting that this age may be differentially sensitive to HPA axis activation after opiate withdrawal. There is a substantial literature indicating that prenatal and early postnatal stress can alter later HPA function, behavior, and neurotransmission, although specific effects are dependent on the type and magnitude of the prenatal or postnatal stress manipulation. For example, restraint stress in pregnant rats augmented the hippocampal acetylcholine response to a mild stress in adult offspring (Day et al., 1998). However, prenatal administration of cocaine, a potent activator of the HPA axis, attenuated the ACTH and corticosterone response to a serotonin releaser in young rats (Cabrera et al., 1994). Thus, because of the consequences of increased corticosterone (e.g., changes in glucose utilization, muscle activity, and blood flow), prenatal withdrawal may be a substantial contributor to the dysfunction associated with prenatal opiate exposure. This is relevant both when withdrawal/detoxification is intentionally brought about, but also when there is repeated cycling of intoxication and withdrawal, characteristic of short-acting street opiates, such as heroin.

**Opiate Withdrawal and Hemodynamic Changes.** The vitelline vasodilation associated with opiate withdrawal and IBMX administration is somewhat counterintuitive based on reports describing opiate withdrawal-associated vasoconstriction, manifest as increased blood pressure and decreased blood flow (Bucafasco, 1983; Chan et al., 1999). The vasoconstriction is presumably caused by enhanced activity of the sympathetic nervous system and is a major sign of opiate withdrawal, with significant clinical implications (Kanof et al., 1992). However, the vasoconstriction described above involves the systemic blood vessels of mature subjects, whereas we examined the extraembryonic vitelline blood vessels, which are analogous to the human placental vasculature (Metcalfe and Stock, 1993). The vitelline blood vessels transmit nutrients from the yolk sac and gas exchange to and from embryo. Interestingly, the vitelline membranes are not innervated, and changes in diameter are regulated by local factors, such as temperature, carbon dioxide content, and the chemical environment (for review, see Romanoff, 1960). We interpret the vasodilation in these extraembryonic blood vessels as a compensatory response to energy and gas-exchange demands associated with withdrawal.

**Mechanisms for Opiate Exposure and Withdrawal Effects.** We had strong evidence that withdrawal from the opiate was increasing serum corticosterone and inducing vasodilation, because chronic NLAAM exposure on its own did not affect these measures. The IBMX studies provided convergent evidence and a possible mechanism for the effects. IBMX is a nonopiate whose administration mimics or exacerbates the behavioral effects of opiate withdrawal (Neal and Sparber, 1986; Holtzman, 1989; Kleven and Sparber, 1989a). In the present studies embryos from eggs treated with IBMX on E18 had increased serum corticosterone and vitelline blood vessel diameter compared with controls, with the time course and magnitude of the responses similar to that seen with the Nxa-precipitated withdrawal. By blocking the action of phosphodiesterase enzymes, IBMX increases intracellular cAMP and subsequent downstream signaling. Because these same signaling pathways are thought to play a role in Nxa-precipitated withdrawal (Christie et al., 1997), it suggests that increased cAMP production is involved in the hemodynamic and neuroendocrine consequences of opiate withdrawal.

**Involvement of 5-HT₂ Receptors.** Pretreatment with ritanserin was effective in preventing the HPA axis and vasodilation associated with both Nxa-precipitated withdrawal and IBMX administration, indicating involvement of 5-HT₂ receptors. Ritanserin is a high-affinity 5-HT₂ receptor antagonist with a long half-life (160 min half-life dissociation in vitro). Its affinity for dopamine₂ and α₁- and α₂-adrenergic receptors is approximately 75- to 170-fold lower than for 5-HT₂ receptors, and the half-life for dissociation from dopamine- and adrenergic receptors is 10–30 min (Leysen et al., 1985). Because ritanserin was administered 16 to 24 h before the induction of opiate withdrawal, it was most likely acting at 5-HT₂ receptors. With respect to changes in the HPA axis, serotonin is an important modulator of feedforward and feedback pathways in the hypothalamus and hippocampus that regulate HPA axis activity after a variety of stimuli (Fuller, 1992). Thus, ritanserin’s actions in this study may have been central in origin, blocking neural 5-HT₂ receptors involved in initiating a corticosterone response. However, it is also possible that blockade of 5-HT₂ receptors in peripheral locations decreased energy, cardiovascular, and muscular demands in the embryo, obviating the need for increased production of glucocorticoid hormones.

Serotonin is also an important hemodynamic modulator (for review, see Martin, 1994), especially in umbilical and chorionic blood vessels, which are reactive to 5-HT₂ agonists both in vivo and in vitro (Marin et al., 1990; Zhang and Dyer, 1990). For example, Marin et al. (1990) found that serotonin was a more potent vasoconstrictor than noradrenaline or histamine in isolated segments of human placental arteries and veins and that this vasoconstriction could be blocked by low doses of the 5-HT₂ antagonist ketanserin. Thus, with respect to blockade of opiate withdrawal-induced vitelline vasodilation, ritanserin’s actions may be directly at the vascular bed. However, as with the HPA axis, ritanserin may be acting to indirectly block other manifestations of withdrawal described above, negating the need for compensatory vasodilation.

Evidence from previous studies in our laboratory, as well as others, have found adverse consequences after hemodynamic and HPA axis alterations in utero or in ovo. Ritanserin is efficacious in blocking these effects, whereas it has no effect on its own or in combination with NLAAM or Nxa. This supports prior findings of no toxicity of “therapeutic” doses of ritanserin during late chick embryonic development (Bollweg et al., 1998; Schrott et al., 1999). Furthermore, it suggests that blockade of 5-HT₂ receptors at these ages may be useful.
therapeutically in situations where excess 5-HT activity is deleterious. However, it is important to note that 5-HT plays multiple roles in the developing nervous system, including acting as a growth and trophic factor during early stages of development (e.g., Lauder and Krebs, 1978). Thus, disruption of 5-HT signaling via receptor blockade, even in the presence of excess 5-HT activity, may have deleterious consequences during certain stages of development (Schrott et al., 1999).

The parallel findings between Nx-precipitated withdrawal and IBMX administration with respect to involvement of 5-HT2 receptors mirror that found for rats, and strengthens the use of the developing chick for further mechanistic investigations. The chick is a popular research tool for development of both form and function. This knowledge can be used to target treatments to ages when specific systems have achieved varying degrees of maturity. Because the chicken is used to target treatments to ages when specific systems have development of both form and function. This knowledge can be used to target treatments to ages when specific systems have achieved varying degrees of maturity. Because the chicken is a precocious species, a wide range of behaviors and physiological indices can be assessed shortly after hatching, in the absence of a maternal or littermate interactions, to further determine consequences of opiate exposure and withdrawal.

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References


Address correspondence to: Dr. Lisa M. Schrott, Department of Pharmacology, University of Minnesota, 6-120 Jackson Hall, 321 Church St. S.E., Minneapolis, MN 55455. E-mail: schro041@umn.edu