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Late Embryonic Ritanserin Exposure Fails to Alter Normal Responses to Immune System Stimulation in Young Chicks

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SCHROTT, L. M., W. A. SWEENEY, K. E. BODENSTEINER AND S. B. SPARBER. *Late embryonic ritanserin exposure fails to alter normal responses to immune system stimulation in young chicks.* PHARMACOL BIOCHEM BEHAV **64**(1) 81–88, 1999.—Prior studies in our laboratory have demonstrated that prenatal treatment with the serotonin$_2$ (5-HT$_2$) antagonist ritanserin is effective in blocking some of the lethal, dysmorphic, cardiovascular, and behavioral consequences of excessive direct or indirect stimulation of 5-HT$_2$ receptors in the developing chicken. The efficacious dose range for ritanserin in these studies had very little or no effect on the above measures of toxicity when administered alone. In the present study, we extend our characterization of ritanserin's potential toxicity, or lack thereof, to include the normal behavioral and endocrine responses to immune system stimulation by the endotoxin lipopolysaccharide (LPS). LPS administration induces a syndrome collectively known as sickness behavior, manifest as altered thermoregulatory processes leading to fever, and increased serum concentrations of neuroendocrine hormones, including corticosterone. These survival-promoting responses to LPS were assessed in young chickens that had been treated with doses of ritanserin ranging from 0 to 2.7 mg/kg on embryonic day 17 (E17). When sickness behavior was assessed in 5–7-day-old chicks 1 h post-LPS injection, E17 ritanserin-treated subjects did not differ from controls. At 4–6 h post-LPS, 4-day-old chicks displayed a robust fever, and E17 ritanserin did not affect the magnitude of this response. Similarly, E17 ritanserin treatment failed to affect corticosterone concentrations 2 h post-LPS in 14-day-old chicks. Thus, ritanserin treatment during late embryogenesis, a time when it is effective against direct and indirect acting 5-HT$_2$ agonists, failed to modify the survival promoting and beneficial interactions between the nervous, endocrine, and immune systems that are elicited following immunostimulation. © 1999 Elsevier Science Inc.

Gallus domesticus Sickness behavior Fever Development Serotonin receptors Chicken embryo Corticosterone Lipolysaccaride

OUR laboratory has a long-standing interest in the potential therapeutic efficacy of serotonin$_2$ (5-HT$_2$) receptor antagonism for blocking the expression of toxic and functional teratogenic effects of exposure to opiates opiate, and quasi-opiate withdrawal, and cocaine in the rat and developing chicken (25,26,34–37,54), because 5-HT may be involved in various aspects of drug abuse. For example, both the potent vasoconstriction (54) and the activation of the hypothalamic–pituitary–adrenal (HPA) axis (30) that accompany cocaine use are mediated by 5-HT receptors. Although the reinforcing efficacy of cocaine (and other abused drugs) has been linked to mesolimbic dopaminergic systems [i.e., (28)], evidence suggests that this system may be modulated by 5-HT neurons (3,12,38). In fact, when the precursor to 5-HT, tryptophan, was depleted acutely, human cocaine users reported a decrease in the subjective "high" to intranasal cocaine (2), and a decrease in cocaine craving following presentation of cocaine cues (42). Thus, both animal and human researchers have attempted to block the intake and/or withdrawal of abused drugs by antagonizing 5-HT receptors. Initial studies in a rat model of forced oral cocaine, alcohol, or fentanyl use found that daily treatment with ritanserin decreased intake of each substance in a dose-dependent manner (21,33) and blocked changes in exploratory behavior following cocaine withdrawal

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(33). However, a subsequent study using intravenous self-administration of cocaine failed to find an effect of ritanserin on a rat's ability to discriminate between cocaine and saline, or on the rate of cocaine self administration (40). Double-blind placebo-controlled studies in humans have also failed to find an effect of ritanserin on cocaine craving (22), or on the "high," and some of the associated physiological responses to the presence of cocaine cues in a laboratory setting (14). However, the latter study found that ritanserin attenuated the decreased skin temperature elicited by cocaine cues, suggesting a role for 5-HT_2 receptors in the vasoconstriction induced by cocaine or cocaine cues.

Although ritanserin has not shown great promise in blocking psychological factors leading to drug abuse, there has been more success in blocking the physiological consequences of drug exposure. In adult rats, expression of both true opiate- and quasi-opiate withdrawal were blocked or attenuated by treatment with various 5-HT_2 antagonists including mianserin, pirenperone, ketanserin, as well as ritanserin (26,34–37). Ritanserin also blocked some of the lethal, behavioral, and vascular consequences of embryonic cocaine exposure [i.e., (25,54)]. These latter studies are important because cocaine exposure during pregnancy has adverse effects on the physiological and neurobehavioral development of the exposed offspring (20,47,52). Although 5-HT_2 receptor antagonists have been used clinically, pregnant women have been excluded from these studies because a dearth of information exists as to their potential toxicity during development. Thus, recent studies from our laboratory have characterized some of the behavioral, neuroendocrine, and cardiovascular effects of mid to late embryonic exposure to the selective 5-HT_2 antagonist ritanserin in chicken embryos and young chicks (5–7,54). These studies have established a "therapeutic" dose range for blocking excessive stimulation of 5-HT_2 receptors, as well as determining the potential developmental toxicity of ritanserin at the effective doses.

Embryonic lethality and vascular disruptions induced by the 5-HT_2 agonist dimethoxyiodophenylaminopropane (DOI) can be blocked or attenuated in a dose-dependent manner by pretreatment with ritanserin in a range of 0.2 to 0.9 mg/kg egg (48,54). The prenatal and postnatal behavioral effects of embryonic DOI exposure can be blocked by administration of 0.3–0.9 mg/kg ritanserin (5,6). Thus, ritanserin's effective dose range for blocking the consequences of excessive stimulation of 5-HT_2 receptors during mid to late embryogenesis in the chicken is in the range of 0.3–0.9 mg/kg. The second issue addressed by these studies was the potential developmental toxicity of ritanserin when administered alone. Ritanserin administered alone at doses of 0.3 to 0.9 mg/kg had no effect on hatching measures, umbilical closure, blood vessel diameter, embryonic motility, or posthatch detour learning when administered on E15, nor on postnatal corticosterone concentrations when administered on E14. However, when ritanserin was administered on E12, the 0.9 mg/kg dose altered posthatch detour learning (6), suggesting that the developmental stage at the time of ritanserin exposure may be an important determinant of its potential toxicity. Thus, for the measures examined to date, the effective dose range displayed minimal to no toxicity, especially during mid to late embryogenesis. However, the potential toxicity of these effective ritanserin doses on neural–immune interactions has not been examined.

It is well established that immune system stimulation by pathogens or endotoxins, such as lipopolysaccharide (LPS), induce a characteristic behavioral pattern known as sickness behavior, which is manifest as decreased food intake, locomotor activity, and grooming; withdrawal from social interactions with conspecifics; altered thermoregulation leading to elevated body temperature; and changes in sleep–wake cycles (13,17). These behaviors are thought to promote survival and are conserved across species [i.e., (13,17,27)]. Thus, as with mammals, chickens display sickness behavior in response to an appropriate challenge (23,24). Immune stimulation also impacts the HPA axis, increasing the plasma concentration of corticosterone and its antecedent releasing hormones. Elevated glucocorticoids act as negative feedback signals, forming a neuroendocrine–immune system loop that helps moderate the immune response [reviewed by (9,43,50)].

Insults to the developing organism have the potential to disrupt the integrated neural–endocrine–immune responses elicited by immune system stimulation. This can occur via altering the normal development of individual systems or by interfering with communication among the systems. Alterations in 5-HT activity might affect LPS-induced behavioral and endocrine responses, because activity at 5-HT receptors influences the expression of many of the same behaviors that are elicited by immune stimulation, such as sleep–wake cycling (45,46), feeding behavior (11,15,32), as well as modulation of the HPA axis (1,4,16,51). In addition, 5-HT is an immunomodulator, and much of the data suggest a biphasic effect of 5-HT on immune function. Both 5-HT depletion (29,53) and increased 5-HT activity suppressed immune function (29,39), while low doses of 5-HT were immunostimulatory (29,53). 5-HT may directly effect immunocytes (18,19,49) or interact with the endocrine system to indirectly influence immune function. Previously, we found that LPS-induced sickness behavior in the chick was diminished by E18 cocaine exposure. Pretreatment with ritanserin was unable to block this effect. There was an indication that ritanserin, when administered alone on E17, affected LPS-induced sickness behavior, although only a single dose of ritanserin was used (44). Thus, the present study was designed to more fully characterize effects of 5-HT_2 receptor blockade during late embryonic development on the behavioral, febrile, and neuroendocrine responses to immune system stimulation by LPS.

METHOD

Subjects

Eggs from a Rhode Island Red × White Leghorn (Babcock substrain) cross were obtained from the Poultry Nutrition Research Center (University of Minnesota, St. Paul, MN). The eggs were kept at 14–16° C for 24–48 h to synchronize embryo development, and were then set in a rotating forced air incubator/hatcher (Humidaire, New Madison, OH). The incubator and hatcher temperature was 37–38° C, and the relative humidity was 56–60%. The day the eggs were set was designated as embryonic day 0 (E0). The eggs were candled on E12–E14, and treatment was assigned randomly to eggs with viable embryos. An injection site for drug administration was marked approximately 1 cm below the air cell, avoiding membrane-bound blood vessels that could be observed during the candling procedure. The shell surface at the injection site was sterilized with a small drop of 2% iodine tincture and wiped with a gauze pad moistened with 70% ethanol to remove the iodine. A 1.2-mm diameter dental burr and a small drill were used to drill injection holes without puncturing the underlying membrane and holes were covered with a small piece of transparent plastic tape (3M, St. Paul, MN). Prior to drug injection on E17, the eggs were candled again and eggs with nonviable embryos were

discarded. The eggs were transferred to the hatcher on E18 and the hatcher was checked twice a day, every 12 h, from E20–E22 for new hatchlings. Hatchlings were removed and banded for identification (day of hatching designated as posthatch day 0). Weight and presence of visibly apparent structural malformations, including herniated umbilici, were recorded and chicks were placed in a heated brooder. Water was provided ad lib upon placement in the brooder and ad lib food (Country Choice Medicated Chick Starter, Land-O-Lakes, Inver Grove Heights, MN) was provided from day 2 forward, except during the food deprivation phase of the sickness behavior assessment. The chicks were placed on a 12 h light:dark cycle with lights on at 0700 h. The sex of the chicks was determined either by identification of the sexually dimorphic comb in older chickens or identification of the internal reproductive organs in younger chicks.

Embryonic Treatment

Ritanserin (Research Biochemical International, Natick MA) or its vehicle, 0.1 M tartaric acid (TA) were administered on E17. In Experiment 1, ritanserin doses of 0.1, 0.3, and 0.9 mg/kg were used, while in Experiment 2 the ritanserin doses were 0.3, 0.9, and 2.7 mg/kg. These doses were based upon previous work in our laboratory (5,6,48). Drug and vehicle solutions were freshly prepared, filtered with 0.2-μm filters (Millipore, Bedford MA), and kept at 4° C. Injections were placed 3 mm beneath the eggshell in a volume of 20 μl. Following the injection, the tape was replaced over the injection hole and the eggs were placed back in the incubator.

Experiment 1. LPS-Induced Sickness Behavior and Elevated Serum Corticosterone

Sickness Behavior. LPS-induced sickness behavior was assessed on posthatch days 5, 6, or 7 using the procedures of our prior study (44), which were modified from Johnson et al. (23,24). The testing was spread over 3 days because of the large number of subjects to be tested ($n = 120$; $n = 30$ from each embryonic treatment group). Treatment groups were equally distributed across test days. Chicks were food deprived (water available ad lib) 24 h prior to testing. One hour prior to testing, 10 chicks from each of the embryonic treatment groups received an intraperitoneal (IP) injection of one of the following, in a volume of 2 μl/g body weight: avian saline (0.85% NaCl), 5 mg LPS/kg, or 15 mg LPS/kg (Sigma, St. Louis, MO; serotype 0128:B12).

Sickness behaviors were observed in a 51 × 61 × 37-cm chamber that was designed for assessing chick detour learning (5). The front and back walls of the chamber were covered with mirrors to decrease isolation-induced distress vocalizations and to facilitate accurate assessment of eye closure. The test room was dark, but the chamber was illuminated by fluorescent lights. A wide angle, low light-sensitive 8-mm video camera was placed in front of the chamber and recorded the session through the front glass panel, which was in reality, a one-way mirror. A Petri dish containing moistened chick food (same as ad lib food described above) was weighed and placed in the center of the chamber. The food was moistened to make it more palatable in the absence of drinking water, and to prevent spread during consumption. The food dish was weighed prior to and following a test session to determine the apparent quantity of food consumed. Just prior to testing each chick was weighed to the nearest 0.1 g. It was then placed in the front right corner of the chamber, facing the Petri dish, at which time the 5-min test session began. At the conclusion of testing, the chick was reweighed to determine if there was a change in body weight. The videotapes were scored by two independent observers blind as to treatment. In addition to determining the amount of food consumed and any changes in body weight, the following behaviors were recorded: 1) number of isolation-induced distress vocalizations, and 2) amount of time spent sleeping, in seconds (cessation of vocalizations, change in respiration and closure of both eyes).

Elevated serum corticosterone. Fourteen-day-old chicks that had not been assessed for sickness behavior were used to determine LPS effects on serum corticosterone. Subjects that received either TA vehicle ($n = 30$) or 0.3 mg/kg ritanserin ($n = 30$) on E17 were treated with either avian saline ($n = 10$ per group), 5 mg LPS/kg ($n = 10$), or 15 mg LPS/kg ($n = 10$). Following injections, the chicks were placed in plastic tubs lined with brown wrapping paper in groups of eight to nine. For 2 h following LPS or saline injection they remained undisturbed in a separate room from where they were to be decapitated. The chicks were rapidly decapitated within 1 min of entry into the animal room where they were kept after injections. Trunk blood was collected, placed on ice, and sera obtained. Sera were stored at −70°C until analysis of corticosterone concentrations. Prior studies (8,10) had determined the appropriate sera dilution for saline-treated chicks and preliminary assays determined the appropriate dilution for LPS-treated subjects. Diluted sera (1:50 for saline-treated and 1:100 or 1:250 for LPS-treated subjects) were heated to denature corticosterone binding globulin and incubated with an antibody directed against corticosterone (ICN Biomedical, Costa Mesa, CA) and ^{3}H-corticosterone (New England Nuclear, Boston, MA). Charcoal was added, antibody bound corticosterone was separated, and bound ^{3}H-corticosterone was counted in a liquid scintillation counter with CytoScint cocktail (ICN Biomedical) to an error of 3%. Standards were run with the samples and sample values determined by interpolation from the standard curve and converted to ng/ml. Samples and standards were run in duplicate, and the average percent coefficient of variation (CV) for standard duplicates was 4.6%.

Experiment 2. LPS-Induced Fever Response

Fever response. The fever response to LPS was determined in 4-day-old chicks. LPS (7.5 mg/kg) or saline treatment was randomly assigned to subjects within each embryonic treatment group. For LPS treatment there were six to eight chicks per treatment group and for saline treatment five to six chicks. Chicks were removed from the brooder in groups of four to six and placed in a plastic tub (24 × 44 × 21 cm) lined with brown wrapping paper. A baseline temperature reading was taken by inserting a small probe (Yellow Springs Instrument Co., Yellow Springs, OH) into the cloaca. The probe was lightly coated with mineral oil, and was connected to a Digitec tele-thermometer that displayed the temperature to the hundredth °C. Following the baseline temperature reading, the chick was weighed, injected with either saline or LPS in a volume of 2 μl/g body weight, and returned to the plastic tub. This procedure was repeated for the remaining subjects in the group, at which point these subjects were then returned to a heated brooder and a new group of chicks removed. Cloacal temperature readings were taken at 2-h intervals until 10 h post-injection. The deviation from baseline temperature in °C was used for analyses.

Data Analyses

The hatching rate was analyzed by chi-square analysis, and the weight at hatching by a one-way analysis of variance

TABLE 1
HATCHING DATA FOR EXPERIMENTS 1 AND 2: EFFECT OF RITANSERIN TREATMENT ON E17

	TA Vehicle	0.1 mg/kg Ritanserin	0.3 mg/kg Ritanserin	0.9 mg/kg Ritanserin	2.7 mg/kg Ritanserin
Experiment 1					
Number (%) hatched	63/67 (94.0)	32/35 (91.4)	64/67 (95.5)	32/35 (91.4)	•
Mean weight ± SEM	41.89 ± 0.37	41.06 ± 0.61	40.28 ± 0.47	40.59 ± 0.62	•
Experiment 2					
Number (%) hatched	41/56 (73.2)	•	46/56 (82.1)	45/58 (77.6)	30/43 (69.8)
Mean weight ± SEM	46.73 ± 0.72	•	46.69 ± 0.74	46.52 ± 0.72	47.20 ± 1.25

(ANOVA), with embryonic treatment as the between-subjects measure. For all experiments, the data were initially analyzed for sex effects or interactions by ANOVA. Because no sex effects or interactions were found, data were collapsed across sex to simplify analysis. In Experiment 1, subsequent analyses were conducted using two-way ANOVA with embryonic treatment and posthatch treatment as the between-subjects measure. In Experiment 2, the cloacal temperature data were analyzed separately within each posthatch treatment group using a one-way repeated-measures ANOVA, with embryonic treatment as the between-subjects measure and postinjection time as the repeated measure. One-way ANOVAs and Fisher's protected least significant difference (PLSD) tests were used for post hoc comparisons among treated groups, and Dunnett's contrasts were used for comparisons between control and treatment groups.

RESULTS

Experiment 1

Hatching data. Chi-square analysis revealed no effect of E17 ritanserin treatment on the hatching rate (Table 1). Ritanserin had a marginal effect on body weight, $F(3, 187) = 2.52$, $p < 0.06$. The ritanserin treated subjects had slightly lower body weights (Table 1). The chicks treated with 0.3 mg/kg ritanserin were significantly lighter than controls ($p < 0.05$; Dunnett's two-tailed test). However, this difference was relatively small (3.8%) and by posthatch day 5 this weight difference had disappeared.

LPS-induced sickness behavior. Due to experimenter error, there were missing data for one subject for body weight and for two subjects for amount of food consumed, so those subjects were excluded from the appropriate analyses. Preliminary analyses revealed no main effects for test day, so the data were collapsed across this variable. A subsequent set of analyses were performed to determine if the dose of LPS affected the degree of sickness behavior. Both 5 mg LPS/kg and 15 mg LPS/kg elicited sickness behavior to what appeared to be a similar degree at the time studied. No significant differences were found between these doses, and the data were collapsed across the LPS dose.

Treatment effects. The data were analyzed by a two-way ANOVA, with embryonic treatment (E17 vehicle, 0.1, 0.3, or 0.9 mg ritanserin/kg) and posthatch treatment (saline or LPS) as the between-subjects measures. Two-tailed Dunnett's tests were used to compare embryonic ritanserin treated groups to the TA vehicle control. No omnibus ANOVA effect of embryonic treatment was found for any of the measures, and all Dunnett's contrasts for embryonic treatment were nonsignificant.

No LPS (posthatch treatment) effect was found for distress vocalizations (Table 2). A significant LPS effect was found for total sleep time, $F(1, 112) = 15.85$, $p < 0.0001$, with LPS

TABLE 2
EFFECT OF E17 RITANSERIN EXPOSURE ON LPS-INDUCED SICKNESS BEHAVIOR; MEAN ± SEM

	Distress Vocalizations	Body Weight Change (g)	Food Consumed (g)
TA Vehicle			
Saline	202.90 ± 56.03	0.60 ± 0.21	0.66 ± 0.25
LPS	186.43 ± 32.20	0.27 ± 0.12	0.45 ± 0.11
0.1 mg/kg Ritanserin			
Saline	158.95 ± 57.91	0.69 ± 0.24	1.01 ± 0.26
LPS	161.88 ± 30.77	0.15 ± 0.11	0.37 ± 0.12
0.3 mg/kg Ritanserin			
Saline	206.65 ± 40.67	0.41 ± 0.17	0.38 ± 0.15
LPS	143.75 ± 32.21	0.38 ± 0.15	0.47 ± 0.14
0.9 mg/kg Ritanserin			
Saline	230.50 ± 61.44	0.45 ± 0.25	0.51 ± 0.17
LPS	190.95 ± 36.76	0.24 ± 0.12	0.32 ± 0.11

$n = 10$ per saline-treated, and $n = 20$ per LPS-treated group.

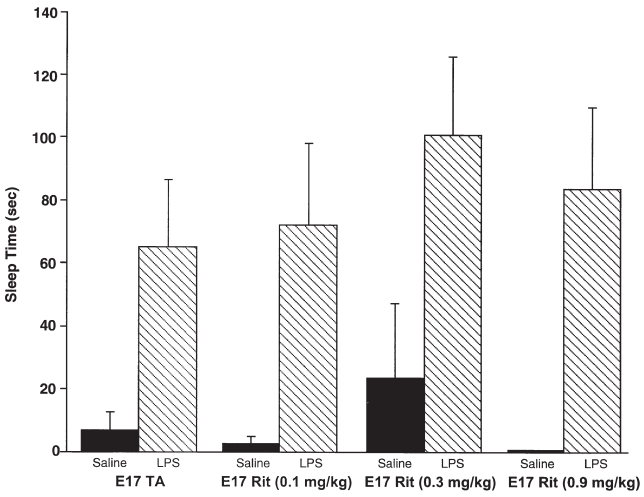

FIG. 1. displays the mean and SEM for the amount of time spent sleeping (seconds) out of a 5-min LPS-induced sickness behavior assessment for 5–7-day-old chicks that received tartaric acid vehicle (TA), or 0.1, 0.3, or 0.9 mg ritanserin/kg (Rit) on E17. One hour prior to testing subjects received avian saline (n = 10 per embryonic treatment group) or LPS (n = 20 per embryonic treatment group).

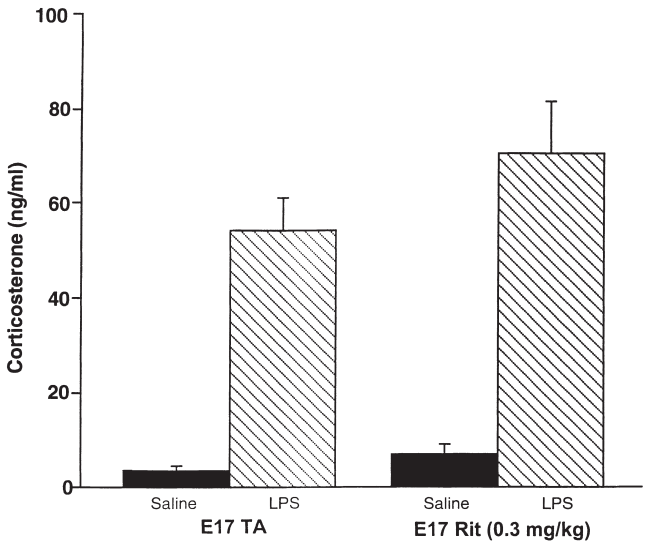

FIG. 2. displays the mean and SEM for the concentration (ng/ml) of serum corticosterone in 14-day-old chicks that received tartaric acid vehicle (TA) or 0.3 mg/kg ritanserin (Rit) on E17. Sera were obtained 2 h following injection with either avian saline (n = 10 per embryonic treatment group) or LPS (n = 20 per embryonic treatment group).

treated chicks spending more time sleeping than controls (Fig. 1). Body weight change was also affected by LPS treatment, with chicks injected with LPS gaining less weight than saline controls, $F(1, 111) = 7.06$, $p < 0.009$ (Table 2; note pretest weight not obtained for one subject). In addition, LPS treated chicks consumed less food than saline treated chicks, $F(1, 110) = 4.86$, $p < 0.03$ (Table 2; note food consumed data not obtained for two subjects). Although the effect was not as large for the amount of food consumed (38% decrease for LPS-treated subjects) compared to the change in body weight (55% decrease in LPS-treated chicks), these two measures were highly correlated ($r = 0.656$, $df = 115$, $p < 0.0001$). The smaller percent change in the apparent amount of food consumed suggests that water evaporation from the moistened food in the Petri dish was negligible. In addition to reduced food consumption, the greater percent decrease in body weight in LPS-treated subjects before and after testing may reflect other physiological processes, such as defecation.

There were no significant embryonic treatment × posthatch treatment interactions, further indicating that ritanserin did not affect the LPS response. However, because the magnitude of the LPS effects may have obscured slight ritanserin treatment effects, for the three measures where significant LPS effects were seen, the data were further analyzed within each posthatch treatment group, a method we used previously to detect effects of embryonic cocaine treatment on LPS-induced sickness behavior (44). A one-way ANOVA, with embryonic treatment as the between-subjects measure, was performed for saline-treated subjects and for LPS-treated subjects. No significant effects of embryonic ritanserin were seen in any of the groups (Table 2).

LPS-induced elevated serum corticosterone. Serum corticosterone concentrations were analyzed by a two-way ANOVA with embryonic treatment and posthatch treatment as between-subjects measures. A significant effect of LPS treatment was found for serum corticosterone concentrations, $F(1, 56) = 38.24$, $p < 0.0001$. As can be seen in Fig. 2, LPS increased corticosterone concentrations substantially (approximately 10-fold) above those of saline-treated subjects. No effects of embryonic ritanserin were found for saline or LPS-treated subjects.

Experiment 2

Hatching data. The overall hatching rate in Experiment 2 was lower than in Experiment 1, likely because the eggs were obtained from an older flock that was near the end of its reproductive capacity. There was no effect of ritanserin treatment on the hatching rate (Table 1). Likewise, there were no effects of E17 ritanserin treatment on hatch weight. Note, however, that the chicks in Experiment 2 weighed about 12% more at hatching than the chicks from Experiment 1.

LPS-induced febrile response. The data were analyzed within the posthatch treatment groups (i.e., saline or LPS). This permitted us to determine if embryonic ritanserin exposure had an effect on basal body temperature (saline treated subjects), as well as on LPS-induced fever. For chicks receiving saline on D4, a repeated measures one-way ANOVA with embryonic treatment (the three ritanserin doses or the TA vehicle) as the between-subjects measure was conducted for the change in body temperature from baseline. There was a significant postinjection time effect, $F(4, 76) = 2.64$, $p < 0.05$, with an increase of approximately 0.25 to 0.30° C in cloacal temperature as the chicks approached their dark cycle. There was no significant effect of E17 ritanserin treatment, nor an interaction between ritanserin treatment and time. All Dunnett's contrasts were nonsignificant. The mean (±SEM) change in temperature for D4 saline-treated groups were as follows: TA vehicle = 0.10° C ± 0.07; 0.3 mg ritanserin/kg = 0.13° C ± 0.09; 0.9 mg ritanserin/kg = 0.06° C ± 0.05; and 2.7 mg ritanserin/kg = 0.32° C ± 0.11.

A similar set of analyses were conducted for subjects injected with LPS on D4. There was a significant effect for postinjection time, $F(4, 104) = 19.89$, $p < 0.0001$, but no effect of embryonic treatment, nor interaction between embryonic treatment and time for the change in baseline temperature. As can be seen in Fig. 3, E17 ritanserin- and vehicle-treated

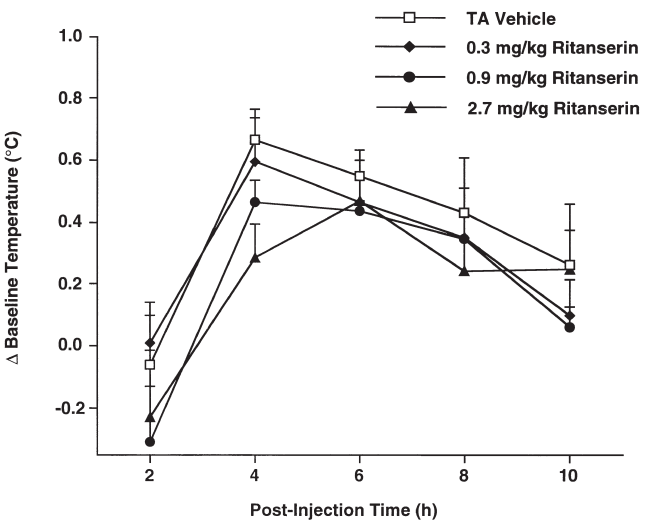

FIG. 3. displays the mean and SEM for the difference from baseline cloacal temperature (°C) following injection with 7.5 mg LPS/kg on posthatch day 4. Chicks had received tartaric acid (TA) vehicle (n = 8), 0.3 mg ritanserin/kg (n = 8), 0.9 mg ritanserin/kg (n = 8), or 2.7 mg ritanserin/kg (n = 6) on E17.

subjects displayed a similar fever response following injection with LPS. The 2.7 mg ritanserin/kg treated group displayed its maximal fever response at a later postinjection time (6 h) compared to the other three treatment groups (4 h). However, Dunnett's contrasts at each postinjection time failed to find any significant difference between the 2.7 mg ritanserin/kg group and the vehicle controls, nor did Fisher's PLSD post-hoc analyses reveal any differences between the ritanserin treated groups.

DISCUSSION

Blockade of 5-HT_2 receptors by ritanserin during late embryogenesis failed to modify the behavioral, neuroendocrine, or fever response to immune system stimulation by LPS. It is important to remember that these responses to LPS are adaptive and thought to enhance recovery and survival during infection with pathogenic agents. The ritanserin doses used in the present study included doses that were effective in blocking the behavioral and physiological effects of excessive embryonic 5-HT_2 receptor stimulation in prior studies [i.e., (5,44,48,54)]. Thus, the failure of embryonic ritanserin exposure to modify the beneficial interactions of the nervous, endocrine, and immune systems further substantiates the lack of toxicity of potentially therapeutic doses of ritanserin in the chick embryo/young chicken model.

One possible reason for our failure to find significant effects of embryonic ritanserin treatment could be that 5-HT systems, specifically 5-HT_2 receptors, involved in the behavioral and neuroendocrine responses to immune system stimulation, have not developed by the late embryonic period when the drug was administered, and thus blockade of receptors failed to affect their development. However, this possibility is unlikely, because detailed mapping studies of the central 5-HT systems in the chick have found that 5HT receptors have developed by E15 (41) and ritanserin is known to have a long half-life (31), which would likely extend into the posthatching period when 5-HT systems are functional. Receptor binding studies with ^{3}H-ketanserin (a 5-HT_2 antagonist) in our laboratory have demonstrated the presence of 5-HT_2 receptors in the E18 chick embryo brain, and that ritanserin can successfully compete for occupation of these receptors, binding with high affinity and a long half-life (25). In addition, prior studies in our laboratory have demonstrated that 5-HT_2 receptors are functional during mid to late embryogenesis, as evidenced by the action of the 5-HT_2 agonist DOI on hatching and behavioral measures, and the ability of ritanserin to block these DOI-induced effects (5,6,48,54). Finally, in a prior study (7), ritanserin was administered on E14 at a dose beyond the effective dose range (2.7 mg/kg), and this dose enhanced stress-induced elevations of serum corticosterone in 19-day-old chickens. Alternatively, the 5-HT systems involved in neural–endocrine–immune system interactions may have developed early in embryogenesis, but were not yet functioning in the perihatching period and, thus, alterations in receptor function may have no apparent effect on posthatch responses.

In general, ritanserin has been well tolerated in both adult animals and humans, with very few observable or measurable consequences in subjects functioning within normal parameters (31), although ritanserin can affect sleep–awake cycling in both humans and rats (45,46) and affect EKG activity in humans (14,22). Thus, an additional hypothesis that is not mutually exclusive with the above, is that blockade of 5-HT_2 receptors during the embryonic period may not have the deleterious consequences that excessive stimulation by selective 5-HT_2 agonists or nonselective agents, such as cocaine, confer on responses to immune stimulation. This hypothesis is supported by the substantial literature detailing a lack of effect of 5-HT_2 receptor blockade on neurological and behavioral measures, while excessive stimulation of 5-HT_2 receptors dramatically alters function. This paradox led Leysen and Pauwels (31); p. 189) to speculate that "5-HT_2 receptors probably received little impetus under normal physiologic conditions. Therefore, acute blockade of the receptor by an antagonist does not disrupt the normal situation, which explains the lack of observable effects by the 5-HT_2 antagonists by themselves." Their data on the state of receptor activation indicate that 5-HT_2 receptors likely exist in a supersensitive state and, thus, blockade may have little functional consequence, while agonists may induce a rapid receptor desensitization. This interpretation is consistent with the present study and our previous work detailing a lack of developmental toxicity for hatching, cardiovascular, behavioral, neuroendocrine, and neural–immune measures when ritanserin doses of 0.1 to 2.7 mg/kg are administered on E15 or later in the chicken embryo. However, data from Bollweg et al. (6,7) suggest that administration of higher doses of ritanserin (e.g., 0.9–2.7 mg/kg) can affect behavioral and neuroendocrine measures in younger (E12–E14) chicken embryos. Thus, the lack of ritanserin toxicity in older embryos may be a function of the maturation of 5-HT_2 receptors, and excessive blockade, like excessive stimulation, may at some developmental stages, lead to dysfunction. This caveat should be considered if ritanserin is to be used in pregnant subjects.

It is important to note that the effects (or lack of effect) of embryonic ritanserin exposure occurred in chicks not exposed to excessive receptor stimulation. This point should be underscored, because if used clinically, ritanserin will likely be given to pregnant women who have excessive 5-HT activity, such as those abusing cocaine or opiates, because these subjects are at risk for a cardiovascular or cerebrovascular accident or neuroimmune dysfunction. Prior work from our laboratory has shown that blockade of 5-HT receptors prenatally can be pro-

tective against the deleterious effects of drug exposure [e.g. (25,44,54)]. Because cocaine is also known to activate the HPA axis, potentially acting as a stressor on the developing fetus, ritanserin's ability to block a cocaine-induced corticosterone surge when coadministered (30) may also be of benefit. Taken as a whole, the present study, as well as other recent work from our laboratory, suggests that embryonic stimulation of 5-HT_2 receptors can affect some physiological and behavioral measures. In contrast, embryonic blockade of 5-HT_2 receptors, at doses that are effective in blocking effects of 5-HT_2 receptor stimulation, have little to no effect on postnatal physiology and behavior, including the survival promoting and beneficial interactions between the nervous, endocrine, and immune systems that are elicited following immunostimulation.

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