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Behavioral and Neuroendocrine Assessment of Ritanserin Exposure in the Developing Chicken: Lack of Toxicity at Effective Doses

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BOLLWEG, G., Y. X. WEI, AND S. B. SPARBER. *Behavioral and neuroendocrine assessment of ritanserin exposure in the developing chicken: Lack of toxicity at effective doses.* PHARMACOL BIOCHEM BEHAV **60**(1) 175–181, 1998.— The 5-HT_2 antagonist ritanserin (RIT) is undergoing Phase III clinical trials for the treatment of substance abuse disorders. RIT has also shown preclinical therapeutic potential for attenuating or blocking lethal and/or toxic effects of exposure to cocaine or the selective 5-HT_2 agonist dimethoxyiodophenyl-aminopropane (DOI) in the developing chicken. To assess the potential toxicity ("side effects") of RIT itself during development, we exposed chicken embryos to 0, 0.1, 0.3, 0.9, or 2.7 mg RIT/kg egg by injecting the drug into eggs with 14-day-old embryos (E14). Voltage generated by spontaneous embryonic activity (motility) was measured on E15 to assess short-term effects of RIT; none were observed. There was no overall effect of these RIT doses on hatchability, though sample sizes were small (n = 13–15 per group). One to 2 weeks after hatching, chicks' acquisition of a detour learning response was tested. There were no observable effects of any RIT dose on detour learning. To assess potential effects of RIT on responsiveness to stress, some chicks were exposed to isolation stress approximately 3 weeks after hatching and killed 15 min later. Blood was assayed for serum corticosterone. There was no effect of any embryonic RIT dose on corticosterone concentrations in nonstressed subjects. Although corticosterone was elevated in all stressed groups, the group exposed to the highest embryonic RIT dose (2.7 mg/kg egg) showed a stress-induced elevation greater than other groups. Thus, except for the highest RIT dose (six to seven times greater than a therapeutically effective dose used in earlier work), embryonic RIT exposure on E14 had no effect on embryonic behavior, hatchability, posthatch learned behavior, and basal serum corticosterone concentrations. At a supraefficacious dose it appears to have modified the responsiveness of the neuroendocrine axis to mild stress. © 1998 Elsevier Science Inc.

Chicken Ritanserin 5-HT_2 receptor Corticosterone

IT has been estimated that approximately 65–75% of human congenital malformations observed during the first year of life result from unknown causes: polygenic, gene–environment interactions; "spontaneous errors" of development; and/or synergistic interactions of teratogens (2). Developmental modifications that later manifest as functional deficits are less apparent than visually observable malformations and may remain undetectable during this period. If origins of functional terata are included, the 65–75% estimate of unknown causes of teratogenesis might require an upward adjustment, given the possibility that many subtle functional alterations may result from environmental or epigenetic insults during development. This possibility has been referred to as "the iceberg under the classical teratological tip" (34).

One factor that may contribute to teratogenicity, functional or dysmorphic, is modified stimulation or blockade of neurotransmitter receptors during nervous system development. Such interactions have been studied in vitro for dopamine (12,35), serotonin, or 5-hydroxytrypamine, 5-HT (7,28,37), and γ-aminobutyric acid (6), while in vivo studies have been reported for these and other transmitters, for example, embryonic nicotine exposure affects choline acetyl transferase and neural activity in young rats (22). Thus, altered function of neurotransmitters and/or their receptors during development may change both short- and longer term nervous system structure and/or function. Because many agents (drugs, toxicants, "stress") affect these interactions, their study is of great potential relevance for teratology.

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In vivo evidence supports the concept that altered 5-HT receptor–ligand intereactions during development can result in long-lasting biological modification. In pregnant rats exposed to the 5-HT reuptake inhibitor fluoxetine (10 mg/kg, SC, E13–E20), decreases were found in hypothalamic 5-$HT_{2A/2C}$ receptor density, and adrenocorticotropic hormone (ACTH) response to the 5-HT_2 agonist dimethoxyiodophenylaminopropane (DOI) was reduced at postnatal day (PND) 70 but not at PND 28 in male offspring (3). This supports the idea that excessive receptor stimulation during development may produce both biochemical and functional deficits later in life. However, other workers (36) exposed pregnant rats to 0–12 mg fluoxetine/kg by daily gavage, then examined functional variables (acoustic startle, locomotor tests, water maze) in progeny at three ages, with and without pharmacologic challenge. No pattern of treatment-related changes in functional variables or regional brain weights was observed in spite of maternal weight loss, decreased litter size and increased neonatal mortality at the highest fluoxetine dose. Thus, simple generalizations regarding the effects of altered 5-HT receptor stimulation during development may not, at least in the short term, be extractable from experimental work that examines varying combinations of species, receptor subtypes, exposure conditions, outcome variables, and drugs like fluoxetine that indirectly affect several receptors.

Study of potential neuroteratogenic consequences of more selective 5-HT receptor manipulation in vivo has also been reported. Synaptic morphology after exposure to a 5-HT_2 agonist (DOI) or antagonist (ketanserin) or their combination during chicken embryonic development has been described (23). Electron microscopic examination of synaptic density in the lateral motor column in chick spinal cord showed dose-dependent, ketanserin-mediated decreases in axosomatic receptor density and DOI-mediated increases in the same measure, although a similar effect after ketanserin plus DOI treatment was also reported, somewhat confounding interpretation. The authors concluded that 5-HT_{2A} receptors are involved in 5-HT modulation of synaptic plasticity. Our laboratory has also examined in ovo and in vivo consequences of 5-HT_2 receptor stimulation (with DOI administration) or blockade (with ritanserin, RIT, a 5-HT_2 antagonist) in the developing chicken, but on other outcome variables (30). DOI reduced embryonic motility, interfered with hatchability, and induced herniated umbilici, effects that were blocked in a dose-dependent manner by RIT. It thus appears that actions at 5-HT_2 receptors, as well as other 5-HT subtypes, for example 5-HT_1 (37), can affect development.

Other work in our laboratory has suggested that the 5-HT_2 antagonist RIT can block lethal effects (e.g., reduced hatchability) or other toxicity (altered embryonic behavior, vasoconstriction) resulting from exposure to cocaine in the developing chicken (10,31,32). These effects are probably due in large part to RIT-mediated 5-HT_2 receptor blockade, which prevents these receptors from binding 5-HT (at synaptic and other sites) made available by cocaine's interference with 5-HT reuptake mechanisms, and attenuates cocaine-mediated changes in neural and/or vasomotor activity. To more selectively examine the potential role of excessive stimulation of 5-HT_2 receptors in such observations, we have investigated RIT's ability to block or decrease toxic or lethal consequences of DOI exposure in the developing chicken, as described above, as well as some posthatch functional effects of such exposure (1).

The purpose of the present experiment was to examine the potential effects of developmental exposure to "therapeutic" and higher doses of the 5-HT_2 antagonist RIT with functional assays. We determined whether injection of RIT during mid–late development into eggs with chicken embryos could affect in ovo behavior (voltage resulting from spontaneous embryonic motility), hatchability, and posthatch behavior (detour learning in 10–15 day old chicks).

For a number of reasons, the present experiment also examined consequences of in ovo RIT exposure in these chicks with an index of neuroendocrine function, serum corticosterone concentration, with or without mild stress. 5-HT appears to modify embryonic form (structure), i.e., to function as a morphogen (13). It is involved in regulating pituitary–adrenal function via serotonergic input to the hypothalamus and pituitary (5), and 5-HT_2 receptors are involved in the regulation of pituitary ACTH secretion (25). Glucocorticoids are an end product of hypothalamic–pituitary–adrenal (HPA) axis activation, and allow adaptation to stressful or threatening conditions by regulating carbohydrate and lipid metabolism, cardiovascular tone, muscle function, immune response, and behavior (the "fight or flight" response). However, maternal stress during development can alter behavioral responsiveness of progeny [e.g., (27)]. 5-HT is involved in the development and regulation of hippocampal glucocorticoid receptors (20), and this process appears to be 5-HT_2 receptor mediated (21). Maternal stress during pregnancy in rats can increase 5-HT_2 receptor number in offspring and modify behavioral responses to a 5-HT agonist (24). Because hippocampal glucocorticoid receptor number may affect negative feedback sensitivity to HPA axis-related stress factors such as corticosterone (18), we determined whether blocking 5-HT_2 receptors during embryogenesis could modify basal or stress-induced serum corticosterone concentrations in early posthatch life.

METHOD

Subjects and Their Treatment

Fertilized eggs with embryos (Rhode Island Red male × White Leghorn female) were obtained from the Poultry Teaching and Research Facility, St. Paul Campus, University of MN. Prior to incubation ("setting"), eggs were refrigerated at 14–16°C for 48 h to synchronize embryogenesis. Eggs were then set in a rotating forced air incubator (Hatchette model, Humidaire Co., New Madison, OH) maintained at ~37.5°C and ~58% relative humidity, and were candled for viability (~80%) on embryonic day 11 (E11; day of setting = E0). Nonviable eggs were discarded. Holes in eggshells for electrode placement (for recording motility related voltages) were drilled approximately 180° apart and approximately 2 cm below the air cell, i.e., halfway along the long axis of the egg (9), with a third hole for drug injections located midway between those for electrodes. Shell surfaces where holes were to be drilled were disinfected with a drop of 2% tincture of iodine, then immediately wiped with a gauze pad moistened with 70% ethanol to remove the iodine. A 1.2-mm diameter dental burr and a small variable-speed drill (Dremel Moto-Tool Model 260, Dremel Mfg. Co., Racine, WI) were used to drill holes, using care to avoid puncturing membranes below the shell. Immediately after drilling, each hole was covered with an approximately 1-cm square piece of transparent plastic tape (3M, St. Paul, MN). Eggs were then numbered and randomly assigned to one of five treatment groups: 0.1 M tartaric acid vehicle, ritanserin (RIT) 0.1 mg/kg egg, RIT 0.3 mg/kg egg, RIT 0.9 mg/kg egg, or RIT 2.7 mg/kg egg.

Drugs and Drug Administration

Drug solutions were prepared the day of the motility experiment and kept chilled on ice until administration. 0.1 M

(+) tartaric acid (tartrate; Calbiochem, Los Angeles, CA) or RIT (a gift from Janssen Pharmaceuticals, Beerse, Belgium) dissolved in tartrate vehicle were administered in 40 μl injection volumes with Hamilton 50 μl syringes (Reno, NV) fitted with a small stop approximately 2.5 mm from the needle tip to ensure standard injection depth. The RIT dose range was selected to include both lower and higher doses than a dose that blocked motility suppressive and lethal effects of cocaine in prior work (0.4 mg RIT/kg egg). To continue the characterization of potential 5-HT-related effects during midembryonic development, eggs were injected with tartrate or RIT the evening before motility was recorded on E15 (i.e., late on E14).

Motility Recordings

Motility was measured on a recording apparatus within an incubator. During recordings eggs were placed on a triangular configuration of phonograph cartridges (11) to minimize transmission of ambient room vibration to eggs. To conduct electric potential produced by embryonic movement, two 28 gauge platinum wire electrodes were inserted approximately 2–3 mm into the holes drilled earlier (9,17). Electrodes were held and positioned with micromanipulators (Model M3301, WPI, Inc., Sarasota, FL). To control for the potential effects of slight age differences at recording time in the rapidly developing chicken embryo, E15 recordings were scheduled such that half were made before, and half after, 1200 h (2400-h clock). Tape was removed from electrode holes that had been drilled on E11 or E12, eggs were placed on the recording stand, and electrodes were inserted. After a 5-min acclimation period, motility was recorded (20 consecutive 15-s "waves" or recording periods, 80 Hz sampling rate, 5 min total recording time). Electrodes were then removed, tape was replaced, and eggs were returned to the incubator.

Electrical signals were amplified 1000-fold with a custom-built preamplifier, then passed through a custom-built low pass filter (low end cutoff frequency = 0.1 Hz; high end cutoff frequency = 12 Hz), then through an analog-to-digital converter (MacADIOS 8AIN, GW Instruments, Somerville, MA, gain = 1). The digital signal was processed and initially analyzed with a commercial wave analysis application (Superscope, GW Instruments, Somerville, MA) run on a Macintosh IIci computer (Apple Computer, Cupertino, CA). Channel function and amplification were confirmed each day before recordings by connecting a device emitting a fixed 3 Hz, 1 mV signal to the electrodes and observing the resulting wave on the visual display grid of a Philips PM3335 oscilloscope (Philips, Enschede, The Netherlands). Voltages detected across the electrodes were processed by the hardware and software and saved as minimum (Min), maximum (Max), Range, and standard deviation (SD; measured in volts) data in spreadsheet format for later statistical analysis.

Motility Statistics

Group mean motility data for Min, Max, Range, and SD of voltage were analyzed with a commercial statistics application (Statview, Abacus Concepts, Berkeley, CA). Motility data are often highly variable and may not be normally distributed, and sample sizes were relatively small for determining this distribution. To reduce the possibility of a type II error (false negative) with regard to possible treatment effects, we avoided this issue by using both parametric tests (one-factor ANOVA followed by Dunnett's test) and nonparametric tests (Kruskal–Wallis, Mann–Whitney *U*) to better allow detection of effects under either assumption.

Hatchability and Body Weight; Posthatch Banding, and Housing of Chicks

On E18 eggs were placed in the hatcher (~37.5°C and ~58% relative humidity) adjacent to the rotating incubator and checked every 8 h for hatchlings on E19–22. All eggs hatched over E20–22. Hatch data were analyzed by chi-square tests. All chicks were weighed shortly after hatching, numbered with small leg bands for identification, then placed in a heated, five-level community brooder with ad lib food and water. Body weight was analyzed by one-factor ANOVA followed by Dunnett's test.

Detour Learning

A few days prior to posthatch day 10 (PHD 10), 12 chicks from each group were selected for assessing potential developmental effects of RIT upon acquisition of a detour learning response. These chicks were separated into two detour learning Test Sets, each kept in a separate brooder (six chicks/group, five groups/Test Set). The night before PHD 10, 12, and 14 (Test Set 1) or PHD 11, 13, and 15 (Test Set 2), chicks were deprived of food in preparation for detour learning assessment. Detour learning has been used as a functional test of nervous system development (26) and for the detection of postnatal consequences of prenatal exposure to drugs [e.g., reserpine (33), cocaine (29) and ethanol (19)] and toxicants such as methylmercury (8).

The detour learning apparatus is a fluorescently illuminated metal enclosure with a hinged lid, separated into two compartments (social and isolation sides) by a clear Plexiglas wall (see Fig. 1). The two sides are connected by two tunnels in the wall, with the same one open throughout the experiment, the other blocked by a Plexiglas barrier. The Plexiglas wall allows isolated subjects to observe those on the social side, while a one-way mirror on one enclosure wall allows the experimenter to observe and record subjects' behavior. To return to the social side, isolated subjects must turn away from the Plexiglas wall (and broodmates on the other side of the wall) and detour through the open tunnel. Under experimental conditions, the opportunity for access to food and broodmates are appropriate stimuli for reinforcing the detour response, resulting in shorter response latency as learning occurs.

On experimental days, five chicks (e.g., one chick from each treatment group in Test Set 1) were randomly selected from the community brooder and placed on the social side of the detour apparatus. Also on the social side was one half of a Petri dish containing a small amount of moistened chick food. Subjects were allowed access to the food and social reinforcement for 30 s, after which one was selected and placed in the center of the isolation side, facing its broodmates on the other side of the Plexiglas wall. The subject was allowed 180 s to face away from the reinforcing complex and detour through the open tunnel. If no detour response was made during this time, its latency was recorded as 180 s, and the subject was gently guided through the tunnel with a wooden ruler, terminating the trial, and beginning another 30 s of food and broodmate access for all subjects. After 30 s the sequence was repeated with a subject from the next group, until each of the five chicks had received four trials. They were then returned to another brooder level furnished with water and ad lib food, and another group of five food-deprived chicks (one from each treatment group) was selected. The procedure was repeated until all 30 chicks in Test Set 1 had completed four trials (1 day); the same procedure was repeated the next day for

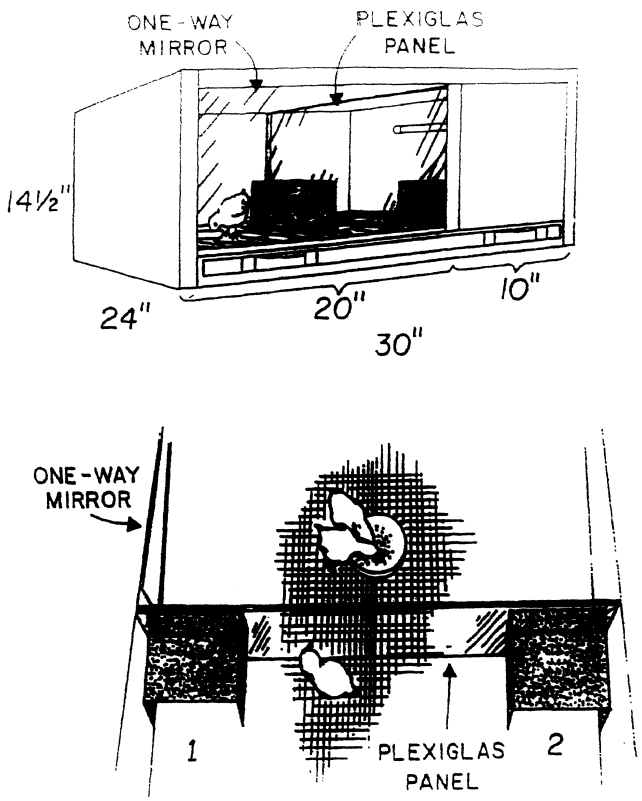

FIG. 1. Detour learning was assessed in a two tunnel apparatus depicted in this figure. Chicks were deprived of food overnight, then allowed access to starter food and social reinforcement for 30 s, after which they were placed behind the Plexiglas partition. They were allowed 3 min (180 s) to face away from the reinforcing complex and detour through one tunnel ("2" in the figure), the other ("1" in the figure) being blocked throughout the experiment. If they did not respond (i.e., detour and emerge from the tunnel within 180 s), their latency was scored as 180 s and they were gently guided through the tunnel with a wooden ruler and allowed access to communal feeding for 30 s. The next chick in the group was then placed on the isolation side of the partition, initiating its trial.

Test Set 2. Time required to test all 30 chicks, as well as the intertrial interval for individual subjects, decreased as the detour response was acquired; i.e., it was greatest on the first day and smallest on the last day of testing. The intertrial intervals also depend upon the number of chicks in each cohort, which in turn depends upon the number of treatment groups. Generally, between three and six chicks comprise a cohort, which may vary from experiment to experiment but is constant within an experiment. The colored, numbered leg bands allowed the experimenter to control for order effects by systematically rotating the sequence of testing for each group of five food-deprived chicks. After every-other-day testing for 6 days (Test Set 1, days 10, 12, and 14; Test Set 2, days 11, 13, and 15), four trials/day, all chicks ($n = 60$) received 12 trials.

Detour Learning Statistics

Response latency (s) was measured with a stopwatch and latency data were analyzed by repeated-measures ANOVA and one-factor ANOVA at each of sessions 1–12. Planned comparisons (RIT treatment groups vs. control) were made with Dunnett's test.

Serum Corticosterone

On PHD 19, chicks from each group were further divided into two groups ("stressed" and "nonstressed"). One nonstressed and one stressed chick, randomly chosen and controlled for order effects, were taken from the brooders at the same time; thus, each dyad contained chicks from different treatment groups. Nonstressed chicks were quietly and quickly removed from their brooder while room lights remained off in the morning, taken to an adjacent lighted room and decapitated for blood collection. Stressed chicks were likewise taken to the adjacent lighted room where they were placed in plastic cages, one chick/cage, and exposed to the ongoing activity in the room. After 15 min these chicks were decapitated for blood collection. Blood samples were allowed to clot in ice-chilled glass test tubes before centrifugation for serum separation.

The serum radioimmunoassay (RIA) for corticosterone was modified from a procedure for rat serum or plasma, with corticosterone antiserum (No. B3-163) raised in rabbit and purchased from Endocrine Science Products (Calabasas Hills, CA). Briefly, the serum sample was diluted 5–10 times with 0.05 M borate buffer/0.25% BSA heated at 60°C for 30 min in a shaking water bath. Fifty microliters of dilute, heat-denatured serum sample was incubated for 45 min with dilute antiserum, [^{3}H]corticosterone and the antibody against corticosterone. The bound radioligand was separated from unbound (free) by precipitating with saturated ammonium sulfate, followed by centrifugation.

Unbound [^{3}H]corticosterone (expressed as a percentage) was determined from supernatant and counted in a Beckman LS583 liquid scintillation counter. A standard curve was constructed for converting the percent unbound ligand in the serum sample to μg percent corticosterone by linear regression. Data were analyzed by ANOVA followed by Dunnett's test.

RESULTS

Motility

Planned comparisons between Tartrate vehicle and RIT-treated groups for motility on E15 are shown below in Table 1. There were no significant differences between vehicle and RIT-treated groups by parametric tests (ANOVA) nor nonparametric tests (overall Kruskal–Wallis test; individual comparisons via Mann–Whitney *U*-test between the 0.1 M tartrate control group and each of RIT 0.1, RIT 0.3, RIT 0.9, and RIT 2.7 mg/kg egg groups).

Hatchability

None of the RIT doses significantly affected hatchability (overall χ^2 (4) = 2.29, $p = 0.68$). Hatchability data are shown in Table 2.

Chicks were weighed soon after hatching and results are shown in Table 3. There was no effect of RIT treatment on body weight, nor were herniated umbilici elevated in the RIT groups: there were two minor herniations in control chicks, one minor and one more distinct herniation in the RIT 0.9 group, and one minor herniation in the RIT 2.7 group.

Detour Learning

Figure 2 depicts detour response latencies during detour learning trials 1–12. Repeated measures ANOVA showed no effect of treatment, $F(4, 55) = 0.26$, $p = 0.90$ or a treatment by trials interaction, $F(44, 605) = 0.74$, $p = 0.89$. However, as indicated by the steep decrease in latency across trials, there

TABLE 1

RIT INJECTED INTO EGGS WITH CHICKEN EMBRYOS ON E14 DOES NOT AFFECT SPONTANEOUS EMBRYONIC MOTILITY ON E15

Treatment	Motility Measure (Mean ± SD, Volts)			
	Minimum	Maximum	Range	SD
0.1M Tartrate	−1.017 ± 0.511	1.037 ± 0.563	2.053 ± 1.070	0.457 ± 0.251
RIT 0.1 mg/kg egg	−1.103 ± 0.357	1.290 ± 0.412	2.393 ± 0.765	0.507 ± 0.196
RIT 0.3 mg/kg egg	−1.104 ± 0.511	1.373 ± 0.821	2.475 ± 1.463	0.521 ± 0.342
RIT 0.9 mg/kg egg	−1.071 ± 0.657	1.182 ± 0.579	2.255 ± 1.008	0.473 ± 0.206
RIT 2.7 mg/kg egg	−0.826 ± 0.434	0.923 ± 0.532	1.750 ± 0.927	0.353 ± 0.211

5 min recording; $n = 6$/group.

was a large repeated measures effect, $F(11, 605) = 35.1$, $p < 0.001$. Similarly, one-factor ANOVA at each of trials 1–12 showed no significant differences between Tartrate- and RIT-treated subjects (data not shown). One-factor ANOVA was also used to analyze the number of "correct" responses (latency <180 s) by four trial blocks (1–4, 5–8, 9–12) in each RIT group. Results showed a lack of treatment effect for trials 1–4, $F(4, 55) = 0.07$, $p = 0.99$, trials 5–8, $F(4, 55) = 0.20$, $p = 0.94$, and trials 9–12, $F(4, 55) = 0.38$, $p = 0.82$. Thus, data were consistent in showing no apparent effect of RIT treatment on detour learning.

Serum Corticosterone

Results of the serum corticosterone (cort) analysis are shown in Table 4.

Two-factor ANOVA showed an effect of stress, $F(1, 20) = 23.25$, $p < 0.01$, RIT treatment, $F(4, 20) = 3.92$, $p = 0.02$, and a stress condition × RIT treatment interaction, $F(4, 20) = 3.16$, $p = 0.04$, for cort concentration. There was no effect of RIT treatment on basal ("nonstressed") cort concentrations by one-factor ANOVA, $F(4, 10) = 0.81$, $p = 0.55$. The mild stress elevated serum cort on average across treatments (0.66 μg cort/dl, nonstressed group vs. 1.11 μg cort/dl, stressed group; $F(1, 28) = 13.47$, $p < 0.01$, and stressed chicks exposed to the RIT 2.7 mg/kg egg dose showed significantly elevated serum cort compared to stressed controls (1.77 vs. 0.79 μg cort/dl, respectively; $t = 3.64$, $p < 0.05$ via two-tailed Dunnett's test).

DISCUSSION

The present results indicate that exposure of developing chicken embryos on E14 (and afterward) to the 5-HT_2 antagonist RIT, at doses shown to block toxic effects of other drugs, had a minor effect on one of a variety of physical and functional outcome measures. Only the dose of RIT (2.7 mg/kg egg), which is up to nine times greater than doses effective against erstwhile toxic doses of DOI or cocaine in embryos and hatchlings of this species, produced evidence of potential toxicity in its own right. This general lack of observed effects was consistent in both pre- and posthatch assays. Interpretation of the detour learning results should include consideration of the timing of this test (posthatch days 10–15). Posthatch days 5–9 were reported as the period of most rapid detour learning (26) and greatest sensitivity for detecting treatment-related learning differences. However, detour learning in chicks tested on posthatch days other than 5–9 is also sensitive to differences between control and treated subjects; e.g., days 7–12 (8); days 7–10 (19); and days 7, 8, 10, and 17 (33). Less ambiguously, there was a clear enhancement of corticosterone responsivity following stress in subjects exposed to the RIT 2.7 mg/kg egg dose, although there was no such effect at lower doses that have blocked or reversed consequences of other drug exposures, e.g., cocaine (10). The RIT 2.7 mg/kg egg dose is nine times that shown to attenuate DOI-induced motility suppression and detour learning alterations [0.3 mg RIT/kg egg; (1)]. Thus, for the measures we examined, RIT appeared to clearly alter only stress related serum corticosterone concentrations, and then only at the highest dose (2.7 mg RIT/kg egg).

E15 embryonic motility showed no effect of any RIT dose injected on E14. We previously reported a motility suppressive effect of the 5-HT_2 agonist DOI (1.0 mg/kg egg) and blockade of this effect by RIT (0.3 and 0.9 mg/kg egg), both injected on E15 (1). We also reported that higher DOI doses (5 and 15 mg/kg egg) injected on E14 reduced viability observed on E16 and also reduced subsequent hatchability (30). Thus, 5-HT_2 receptors appear to be present and functional by E15, but motility and viability may be more sensitive to stimulation than blockade of this receptor subtype.

No dose of RIT in the present study significantly affected hatchability. However, there is a strong suggestion of such an effect in the RIT 2.7 mg/kg egg group, in which 80% of sub-

TABLE 2

E14 INJECTION OF RIT (0.1–2.7 mg/kg EGG) INTO EGGS WITH EMBRYOS DOES NOT AFFECT HATCHABILITY

Treatment	Hatchability
Tartrate 0.1 M (40 μl/egg)	14 of 15 (93%)
RIT 0.1 mg/kg egg	12 of 13 (92%)
RIT 0.3 mg/kg egg	13 of 14 (93%)
RIT 0.9 mg/kg egg	14 of 15 (93%)
RIT 2.7 mg/kg egg	12 of 15 (80%)

TABLE 3

E14 INJECTION OF RIT (0.1–2.7 mg/kg EGG) INTO EGGS WITH EMBRYOS DOES NOT AFFECT HATCHLING BODY WEIGHT

Treatment	Body Weight (Mean ± SD, g)
Tartrate 0.1 M (40 μl/egg)	46.6 ± 4.9
RIT 0.1 mg/kg egg	43.2 ± 4.2
RIT 0.3 mg/kg egg	44.6 ± 4.9
RIT 0.9 mg/kg egg	45.1 ± 3.9
RIT 2.7 mg/kg egg	42.9 ± 3.3

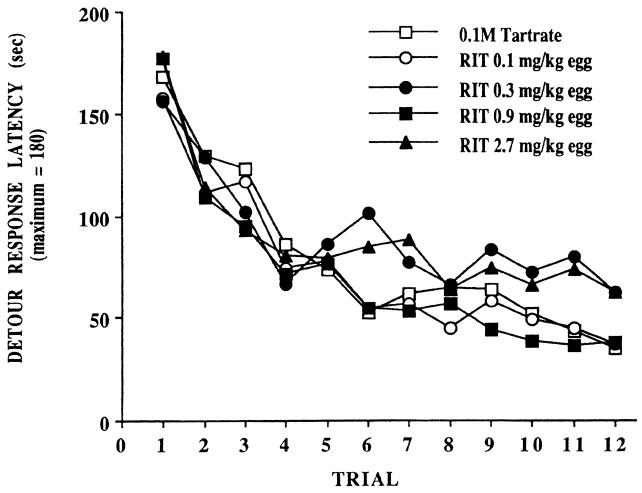

FIG. 2. Detour learning response acquisition latencies for 10–15-day-old chicks. On embryonic day 14, eggs with embryos were injected with RIT 0.1–2.7 mg/kg egg or Tartrate vehicle. RIT did not affect detour learning at any dose tested. Each point represents the mean detour response latency on that trial for a group of 12 chicks.

jects hatched, compared with 92–93% in the other RIT groups. If these hatch proportions occurred in a similar experiment with larger sample sizes (>50 subjects/group), 80% vs. 93% hatchability would have been a statistically reliable reduction. As stated above, body weight soon after hatching was not affected by embryonic RIT exposure at these doses.

Several observations appear to be supported by the corticosterone (cort) assay data. First, the direct RIA method used for chicken serum cort was sensitive and reproducible, with basal serum cort measured in 2–3-week-old chicks ~0.66 μg%, consistent with literature reports [e.g., (4,16)]. Second, E14 RIT exposure in chicks did not affect basal posthatch serum cort concentrations. Third, injection of 2.7 mg RIT/kg egg on E14 enhanced a posthatch cort response to mild stress, which may be partly due to a role of 5-HT and 5-HT_2 receptors in the developmental regulation of cort receptor density. This speculation is consistent with findings in male rats exposed in utero to the 5-HT reuptake inhibitor fluoxetine, in which hypothalamic 5-HT_2 receptor density was decreased (3), and consistent with findings already cited (20,21). Because these receptors may facilitate negative feedback sensitivity to cort, their reduced number could allow an exaggerated and persistent cort response. However, several cautions are appropriate in interpreting the effects of the 2.7 mg RIT/kg egg dose. In rat and guinea pig brain, RIT interacted with other receptors in addition to those of the 5-HT_2 subtype [i.e., histamine H_1, alpha-adrenergic, dopamine D_2 (14)], although its affinity for 5-HT_2 sites was much greater and more prolonged. Thus, the likelihood of non-5-HT_2 effects of RIT are greater at higher doses, e.g., the 2.7 mg/kg egg dose in the present work. Given the small sample sizes (n = 3) in the cort assay, another potentially relevant factor is the chicks' gender, which was not determined in these experiments and which could have skewed the data. This is partly mitigated by results from another experiment indicating that serum cort concentrations in male and female control chicks with treatment histories similar to those in the present experiment did not differ (unpublished observations). An additional caution in interpreting the effects of the highest dose in the present results is that RIT's actions in the developing chick may differ from those in other developing species (e.g., rat). Finally, postnatal neuroendocrine function (basal cort concentration and response to stress) did not appear affected by RIT doses shown to protect against DOI-induced herniated umbilici and effective against cocaine's lethal effects in the chick embryo (i.e., <2.7 mg/kg egg).

TABLE 4

SERUM CORTICOSTERONE CONCENTRATIONS UNDER DIFFERING STRESS CONDITIONS IN 19-DAY-OLD CHICKENS AFTER E14 EXPOSURE TO VEHICLE OR RIT

	Serum Corticosterone Concentration (μg/dl ± SD; n = 3/Group)	
Treatment	Nonstressed	Stressed
Tartrate 0.1 M (40 μl/egg)	0.65 ± 0.04	0.79 ± 0.15
RIT 0.1 mg/kg egg	0.59 ± 0.10	1.04 ± 0.19
RIT 0.3 mg/kg egg	0.75 ± 0.06	0.89 ± 0.10
RIT 0.9 mg/kg egg	0.59 ± 0.13	1.08 ± 0.44
RIT 2.7 mg/kg egg	0.73 ± 0.28	1.77 ± 0.54*
Mean	0.66	1.11†

*$p < 0.05$ vs. stressed tartrate 0.1 M via ANOVA and two-tailed Dunnett's test.

†Significantly greater than nonstressed mean via ANOVA, $F(1, 28) = 13.47$, $p < 0.01$.

Although we report few consequences of RIT exposure under the conditions of this experiment, it is possible that the chicken embryo may be more sensitive to RIT at other developmental periods for the present outcome variables, and that species other than the chicken may be more or less sensitive to such drugs during mid-development. In addition, end points other than those we examined (e.g., modified enzymatic function) could also reveal RIT treatment effects. For example, earlier work from our laboratory (15) examined whole brain tyrosine hydroxylase (TH) activity and catecholamines in 3- and 29-day-old chicks following injection of eggs with the catecholamine-depleting drug reserpine prior to incubation, or into the yolk sac on E7 or E14. In 3-day-old chicks, TH was elevated if reserpine was injected before incubation, but not if it was injected on E7 or E14; whole brain catecholamines were decreased after all three injections. In 29-day-old chicks, TH activity was elevated in the preincubation injection group and not elevated in the E7 or E14 injection groups (same pattern as 3-day-old chicks); however, whole-brain catecholamines were elevated in the preincubation injection group and not different from controls if reserpine was injected on E7 or E14. Thus, sensitive or "critical" periods, other than or after E14 may exist for dysfunctional consequences of excessive 5-HT_2 receptor blockade. Additional studies are underway to examine this possibility.

In summary, we report no evidence of short-term and longer term consequences of injecting eggs with 14-day-old embryos with the 5-HT_2 antagonist RIT at doses shown effective in earlier work against toxic effects of direct or indirect 5-HT_2 agonists. These data support the notion that RIT is safe at doses, which are efficacious against dysmorphic and dysfunctional teratogenic doses of DOI or cocaine exposure late during embryogenesis of the domestic chicken.

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