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Excessive Stimulation of Serotonin$_2$ (5-HT$_2$) Receptors During Late Development of Chicken Embryos Causes Decreased Embryonic Motility, Interferes With Hatching, and Induces Herniated Umbilici

S. B. SPARBER,*[1] A. RIZZO† AND B. BERRA†

*University of Minnesota, Department of Pharmacology, 435 Delaware Street SE, Minneapolis, MN 55455
and †University of Milan, Milan, Italy

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SPARBER, S. B., A. RIZZO AND B. BERRA. *Excessive stimulation of serotonin$_2$ (5-HT$_2$) receptors during late development of chicken embryos causes decreased embryonic motility, interferes with hatching, and induces herniated umbilici.* PHARMACOL BIOCHEM BEHAV 53(3) 603–611, 1996. – The existence and functional significance of 5-HT$_2$ receptors in chicken embryos was studied by injecting the selective agonist dimethoxyiodophenylaminopropane (DOI), alone or in conjunction with the selective 5-HT$_2$ antagonist ritanserin (RIT), into domestic chicken eggs with embryos of varying ages. DOI caused dose-dependent reductions in hatchability and herniated umbilici in hatchlings. These effects were observed after injection early, mid, or late during embryonic development, with evidence of the toxic effects of DOI being greater in older embryos, probably due to 5-HT$_2$ receptor activation late in development, even after injecting DOI as early as on day 3 of embryogenesis. This is based upon the fact that embryos in eggs injected with DOI early continued to develop apparently normally, failing to hatch, often after pipping their shells. Additionally, those that hatched often did so with herniated umbilici, as did late-exposed embryos, indicating that DOI's effects upon this organ were most likely mediated during the prehatching period (i.e., days 18–20). The agonist's selectivity was confirmed by the capacity of RIT to dose dependently block both of these toxic effects of DOI. Reduced embryonic motility monitored on day 19, after injection of DOI on the evening of day 18, suggests that excessive activation of 5-HT$_2$ receptors late during development of this species interferes with some normal embryonic behaviors and physiological changes neccessary for inducing and/or maintaining the hatching process.

Serotonin$_2$ receptors Chicken embryo DOI Development Ritanserin

THE concentration of aminergic transmitters, including serotonin (5-hydroxytryptamine, 5-HT), has been manipulated in the developing nervous system with precursors or with various pharmacological agents known to affect their synthesis, storage, and metabolism. Such studies have been carried out with embryos and fetuses of various species, including the domestic chicken (3,20,21,35). Functional experiments have indicated that manipulating one or more of these parameters may cause behavioral effects postnatally in this species (19,34,35) as well as in the rat (36). Although there is a very early appearance of cells containing 5-HT in the chick embryo nervous system, their pattern of development and terminal thickenings suggest a later, rather than earlier function vis-à-vis synaptic transmission (26,30,31). In addition, 5-HT has been demonstrated to have trophic properties in the rat, so that manipulating its concentration during early development can lead to altered

[1] To whom requests for reprints should be addressed.

patterns of cellular migration and synaptic connectivity (16, 38), which may be related to some of the functional postnatal changes alluded to above.

Many psychotropic drugs can indirectly affect 5-HT neurotransmission and/or other physiological functions in which this indoleamine is involved (e.g., vasomotor tone and gastrointestinal motility). Moreover, the expression of opioid-type withdrawal in rats can be enhanced by administration of 5-HTP, the immediate precursor of 5-HT, and blocked or attenuated by administration of drugs like RIT and mianserin (10,23–25). This indicates that 5-HT_2 receptors are activated during opioid-type withdrawal and are involved in its expression. Interestingly, the capacity of chicken embryos to express opioid or quasi-opioid withdrawal prior to days 12–14 of embryogenesis is very limitted (1,33). Because 5-HT (via 5-HT_2 receptors) seems to be intimately involved in the expression of opioid-type withdrawal, and because the 5-HT pathways or fiber tracts in the CNS of the developing chicken embryo are still primitive at early to mid stages of development, it seems logical to hypothesize that administration of drugs which act through 5-HT_2 receptors would not (adversely or otherwise) affect embryonic development until the receptors are expressed and/or they are functional.

Some of cocaine's acute pharmacological or toxicological effects may also be attributable to altered serotonergic activity via its capacity to potently block the reuptake process for 5-HT (27,28), thereby causing excessive activation of one or more of its receptors. Thus, effects of cocaine administered during development may be mediated indirectly, at least in part, by excessive amounts of 5-HT acting as a transmitter and/or as a trophic factor during sensitive periods. This possibility is supported by observations that reduced motility and hatchability caused by injection of cocaine into chicken eggs on day 18 of embryogenesis can be antagonized by prior injection of RIT, at a dose that itself may not affect embryonic motility and at a time when it significantly reduces available occupation sites for 5-HT_2 ligands (i.e., downregulates these receptors) in embryonic chick brain [(2,17), Kim and Sparber, submitted].

Therefore, to more directly characterize the functional significance of 5-HT_2 receptor activation during various stages of chicken embryonic development and to confirm the possibility that opioid withdrawal or acute cocaine administration can, indeed, act to significantly affect development by (indirect) excessive stimulation of these receptors, we injected DOI into incubating eggs on days 3, 14, or 18 of embryogenesis, day 0 being the first day of incubation. DOI has been demonstrated to be a selective 5-HT_2 agonist (5) and, thus, we attempted to antagonize one or more of its effects by pretreatment of the embryos with RIT in some experiments.

The recent acknowledgement of multiple 5-HT_2 receptor subtypes, based upon sequence homology, overlapping affinity for most ligands, and the use of the same second messenger system suggests further selectivity vis-à-vis developmental pharmacological characteristics. However, DOI and/or RIT do not discriminate sufficiently between the 5-HT_2 receptor subtypes for us to determine which 5-HT_2 receptor is responsible for the various effects we report upon herein. Thus, we do not differentiate between the two or more subtypes of these receptors in this manuscript, refering to them generically as 5-HT_2 receptors.

METHOD

Subjects

Fertile chicken eggs (White Leghorn, Ross strain) were obtained from a local hatchery (Incubatorio Bergamasco s.r.l., Bergamo, Italy). For electrical recording of embryonic motility, two holes were drilled 180° apart at the midpoint of the long axis of the egg for electrode placement (9). For drug injection, a third hole was made half-way between the first two holes and approximately 2 cm from the aircell, along the long axis of the eggs. The procedure for preparing the eggs for drilling, recording embryonic motility and injecting drugs beneath the eggshell has been described in detail elsewhere (33). For calculating doses and ease of injection the eggs were assumed to weigh 50 g, although generally they were about 2–5 g heavier. They were set in a forced-air incubator (Incubatrici FIEM, Como, Italy) and maintained at 37.5°C and 58–60% relative humidity. The first day of incubation was considered day 0 of embryogenesis. Random assignment to treatment groups was maintained throughout. If an eggshell cracked during incubation (due to mechanical turning and/or tightness of fit in the rack), it was discarded from the experiment.

Drugs

(±)-1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane HCl (DOI) was purchased from Research Biochemicals Incorporated (Natick, MA) and dissolved in avian isotonic saline (0.85% NaCl solution). Ritanserin was kindly supplied by Janssen Research Foundation (Beerse, Belgium) and dissolved in tartaric acid. Solutions were made fresh just prior to injection and passed through a 0.2 μm filter for cold sterilization. Doses are expressed as the base. All injected volumes were 44 μl.

Embryonic Motility

For recording embryonic motility, an egg was placed on a triangular arrangement of three phonograph needle cartridges to minimize vibrations and contact with other surfaces (11). Two platinum wire electrodes (28 gauge) were inserted approximately 3 mm into the holes to conduct the electrical potential produced by embryonic movements (9). The electrodes were inserted with micromanipulators and the embryo was allowed to acclimate for 3 min, at which time 5 min of spontaneous motility was recorded. The signal was split and recorded with an analog recorder (Model N2, Gilson Medical Electronics, Middleton, WI) or amplified by a factor of 500 with an AC/DC preamplifier (Model 50-5131, Harvard Apparatus, Inc., South Natick, MA). The amplifier contained band-pass filters that were set to filter frequencies below 1 Hz and above 40 Hz. The amplified signal was passed through an analog/digital converter (MacADIOS 8ain, GW Instruments, Somerville, MA), which was connected to a Macintosh computer with a data collection and processing program (SuperScope, GW Instruments). The sampling rate was at 100 Hz and we analyzed various parameters of the signal, including minimum, maximum, and standard deviation of the voltages for each sweep, which lasted 5 s. Thus, data for each parameter were sent to the SuperScope journals set up for each embryo. These, in turn, were copied to Statview (Abacus, Berkeley, CA) files for statistical analyses.

Viability, Hatchability, and Herniated Umbilici

Eggs were candled at various times during the incubation period, depending upon the day of injection with DOI or its vehicle. We were interested in determining if DOI injected during early or midembryogenesis caused a reduction in viability within a few days of the injection, or if the embryos remained viable until they were preparing to hatch. A pilot ex-

periment suggested that the latter would most likely be the case and that DOI would possibly cause herniated umbilici in the hatchlings. As such, we decided to allow the hatchlings' down to dry and fluff out for up to several hours before removing them from the incubator after they hatched. At the time of removal from the incubator we determined if there was a herniated umbilicus and used a vernier caliper to measure the approximate diameter of the herniated umbilici. We have not included the 1–2 mm remnant of an umbilicus that was not resorbed or that did not fall off by itself. In other words, 2 mm was subtracted from the measured sizes of the umbilici of the hatchlings and these data were subjected to statistical analyses. When RIT was injected, it was done 3 h prior to DOI, late enough in development (day 18) so as to preclude checking them for viability, but relying upon hatchability as the measure of its potential protective effect against DOI, or of its own potential toxicity vis-à-vis hatchability.

Statistics

Viability, hatchability, and the frequency of herniated umbilici data were analyzed using Chi-square analyses. Chick body weights at the time of hatching and the size of herniated umbilici were analyzed using a one-factor ANOVA, followed by the Dunnett's or the protected Least Significant Difference test, as appropriate. Because of the nature of the distribution of data derived from measuring embryonic motility, the Mann–Whitney test was utilized. An alpha level of 0.05 was set for concluding there were significant treatment effects.

EXPERIMENT 1. INJECTION OF DOI ON DAY 3 OF EMBRYOGENESIS: EFFECTS UPON VIABILITY ON DAYS 6 AND 13 AND UPON HATCHABILITY

Previous studies have indicated that drugs or other chemicals injected into the yolk or just beneath the shell of chicken eggs that contain developing embryos are absorbed into the circulation, or in other ways gain entrance to the embryo within minutes to a couple of days after injection (8,13,33,37). Accordingly, an initial dose-ranging experiment was undertaken to determine if DOI's effects were likely to be manifest as a dose-dependent reduction in viability shortly after injection on day 3 of embryogenesis. For example, if the expression of opioid or opioid-like withdrawal in the chicken embryo is dependent in great part upon the presence of functional 5-HT$_2$ receptors, as we suspect, we should not expect to observe dramatic toxicological effects of DOI upon viability until sometime near or after the end of the second trimester of development of this species. This was hypothesized because we have been unable to demonstrate a robust expression of opioid withdrawal in embryos younger than 14 days of age. Moreover, quasi-opioid withdrawal caused by an injection of isobutylmethylxanthine into embryos on days 12–14 of embryogenesis was not observed if injected earlier (1). Embryos continuously exposed to an opioid starting shortly after its injection on day 3 embryogenesis (13) did not express robust withdrawal upon injection of the opioid antagonist naloxone on day 14 [(33), Sparber, unpublished observations], even though expression of withdrawal later during embryonic development or shortly after hatching in this model of opioid dependence is unequivocal. Modest withdrawal has been shown to be responsible for changes in brain ornithine decarboxylase activity, rather than exposure to the opioid (dependence) per se, in the absence of withdrawal (14). In its severest form, withdrawal shortly before hatching leads to death of the embryos in ovo (15). Spontaneous neonatal withdrawal is most likely responsible for postnatal behavioral changes we have observed in chicks that have hatched from eggs injected with a long acting opiate (12).

We have previously attributed these observations to the possibility that the biological bases for the expression of withdrawal in this species is not developed sufficiently to be expressed during early stages of embryonic development. Thus, if 5-HT$_2$ receptors were not present and/or functional until later stages of development, DOI should not produce noteworthy effects in young embryos.

Results and Discussion

After injection of saline, 2.5, 12.5, or 25 mg DOI/kg of egg on day 3, 87–97% of the embryos were alive on day 6 (n = 22–30/group, sometimes larger groups were injected with higher doses of DOI because of expected reductions in viability or hatchability, space in the incubator permitting). Upon candling on day 13 we determined that there was an additional small reduction in viability, with 78–87% alive at this stage. However, Chi-square analyses indicated no significant treatment effect of DOI upon viability during the 10 days following injection on day 3.

In order for hatching to occur, a series of complex behaviors are engaged in, including the act of membrane penetration, pipping the shell, followed by convulsive-like climactic kicking to split the shell, allowing the hatchling to emerge (7). Of the 53 embryos injected on day 3 that pipped a hole in the shell, only 6 of them failed to complete the hatching process, indicating that at least this component (i.e., pipping) of the complex hatching behavioral repertoire was apparently intact. When these data (i.e., alive on day 13 and started the hatching process) were subjected to Chi-square analyses, an overall significant effect of DOI was evident, $\chi^2(3) = 15.23, p = 0.002$. Of the control group alive on day 13, 94% (17 out of 18) pipped a hole in their shells. The low, medium, and high dose DOI groups pipped their shells at frequencies of 82% (14 out of 17), 50% (10 out of 20), and 46% (12 out of 26), respectively. Subsequent analyses indicated that the medium and high doses of DOI injected on day 3 significantly reduced the incidence of pipping [12.5 mg/kg, $\chi^2(1) = 9.10, p = 0.003$; 25 mg/kg, $\chi^2(1) = 10.94, p = 0.001$]. Similar analyses for hatchability of embryos alive on day 13, as would be expected from the pipping data, indicated an overall reduction in completing the hatching process, $\chi^2(3) = 12.41, p = 0.006$. Of the saline-injected controls alive on day 13, 83% (15 out of 18) eventually hatched. The low dose DOI group had 76% (13 out of 17) hatch, while the medium and high dose groups had 40% (8 out of 20) and 42% (11 out of 26) hatch, both being significantly reduced [medium: $\chi^2(1) = 7.45, p = 0.006$; high: $\chi^2(1) = 7.41, p = 0.007$]. Only two controls, one of the low dose, two of the medium dose, and one of the high dose embryos failed to emerge from their shells within 2 days after hatching began. Therefore, from this initial experiment, it was obvious that practically all of the embryos that pipped a hole in their shells eventually went on to successfully complete the hatching process, although DOI dose-relatedly reduced hatchability when injected on day 3 of embryogenesis. We can also assume that factors other than DOI (e.g., seasonal effects, drilling holes in the shell, using the small, commercially available incubator for the first time, etc.) were responsible for the 15–20% reduction in viability by day 13, which was evenly distributed amongst the four groups. Therefore, by assigning a value of 100% to the control group, hatchability for the low, medium, and high dose DOI groups was 92, 48, and 51%, respectively, compared with controls (Fig. 1).

It is possible that a drug-induced delay in hatchability

could indirectly reduce overall hatchability because the outer membrane just beneath the shell tends to dry out and become leather-like as the hatching process is prolonged. This, in turn, would make it more difficult for the hatchling to emerge from the shell, having developed apparently normally prior to the hatching process. Chi-square analyses of the hatchlings that were alive as embryos on day 13 and hatched during the first 24 h after the first chick hatched out of the shell indicated an overall significant effect of treatment on this parameter as well, $\chi^2(3) = 14.01$, $p = 0.003$. Of the controls alive on day 13 that eventually hatched, 50% did so during the first 24 h. Of the chicks in the low, medium, and high dose DOI groups, 35, 10, and 8%, respectively, hatched during this period. Thus, the medium and high dose groups had significantly fewer chicks hatch during the first 24 h [12.5 mg/kg, $\chi^2(1) = 7.37$, $p = 0.007$; 25 mg/kg, $\chi^2(1) = 10.15$, $p = 0.001$]. The delayed hatching effect of DOI was evidently responsible for, or in other ways associated with, the overall reduction in hatching in the medium and high dose groups, because there was no evidence of a drug-induced effect upon viability on day 13 of embryogenesis. In fact, when unhatched embryos (i.e., those with pipped and unpipped shells) were examined 3 days after hatching commenced (i.e., after it was apparent that no more hatchlings would emerge), most were observed to have developed to a stage equivalent to at least 19-20 days of embryonic age (6). Of the DOI-exposed chicks that hatched, many were observed to have either herniated umbilici or, in a few instances, they hatched with unresorbed yolk and portions of their intestines externalized. These chicks tended to be runted and died or were euthanized soon after hatching.

The results of this first experiment supported our hypothesis that the presence of a 5-HT_2 agonist during early or mid embryogenesis had little or no effect, measured as embryonic viability. It is consistent with the idea that 5-HT_2 receptors were not present and/or sufficiently functional until sometime after days 12-13 of embryogenesis and that excessive activation of these receptors later during development interfered with hatching by killing the embryos directly, by delaying the hatching process, thereby killing them indirectly, and/or by causing severe constriction of smooth muscle (e.g., umbilical vessels or intestines) preventing resorption of yolk, closure of the abdominal wall, and as yet other unknown toxicological effects responsible for the delayed or reduced hatchability and the herniated umbilici we observed in hatchlings. The lack of effects of DOI upon viability during early embryogenesis is in contradistinction to that observed by Kuwahara and Sparber (13), who observed a reduction in viability within a day after injecting the opiate l-a-acetyl-normethadol at the same age, via the same route.

EXPERIMENT 2. INJECTION OF DOI ON DAY 14 OF EMBRYOGENESIS: EFFECTS UPON VIABILITY ON DAY 16 AND UPON HATCHABILITY

The second experiment was carried out to determine if injection of DOI at a later stage of development, at a time when 5-HT pathways are approaching maturity and the embryo can start to express opioid-type withdrawal, would cause similar effects and if the embryos' sensitivity to the 5-HT_2 agonist was enhanced or diminished with advancing age. It is also possible that early exposure (i.e., sometime between day 3 and 13 of embryogenesis) was necessary for the toxic effects we observed, even though their manifestations did not emerge until shortly before and during the hatching process. This experiment was carried out to address this possibility as well.

In this experiment we injected doses of DOI that were lower and higher than the medium dose used in the first experiment (i.e., 12.5 mg/kg of egg). That dose caused a significant reduction in hatchability when injected on day 3 of embryogenesis. Thus, 5 or 15 mg DOI/kg of egg were injected on day 14, viability was checked on day 16 of embryogenesis, and hatchability determined starting on day 20, when the chicks began to hatch.

Results and Discussion

When saline, 5, or 15 mg DOI/kg were injected into 18-19 eggs with viable embryos on day 14, there was a significant reduction in viability on day 16, $\chi^2(2) = 7.20$, $p = 0.027$. The low dose reduced viability by 21%, $\chi^2(1) = 4.47$, $p = 0.035$, while the high dose reduced it by 33%, $\chi^2(1) = 7.56$, $p = 0.006$. None of the eggs injected with avian saline died during these 2 days. Furthermore, while all of the saline injected eggs went on to hatch (Fig. 2), there was an overall treatment effect, $\chi^2(2) = 10.79$, $p = 0.005$. Only 63% [$\chi^2(1) = 8.58$, $p = 0.003$], and 56% [$\chi^2(1) = 10.77$, $p = 0.001$], of the low and high dose DOI groups, respectively, hatched. These data strongly support the notion that 5-HT_2 receptors are present and sufficiently functional sometime shortly after day 13 of embryogenesis (e.g., days 14-16), so that excessive activation of them leads to significant reductions in viability even before they proceed to the hatching phase of development. These data, together with the data from the first experiment, indicate that DOI may not be metabolized to a great extent, if at all, by the young embryo and doses that are acutely toxic (i.e., lethal) when injected sometime after the 13th day of embryogenesis are apparently not acutely toxic when injected earlier (e.g., day 3). Moreover, as in the first experiment, we continued to observe substantial numbers of hatchlings with herniated umbilici in this brood of survivors exposed to DOI. Therefore, in subsequent experiments we planned to do a more systematic assessment of the incidence and size of this

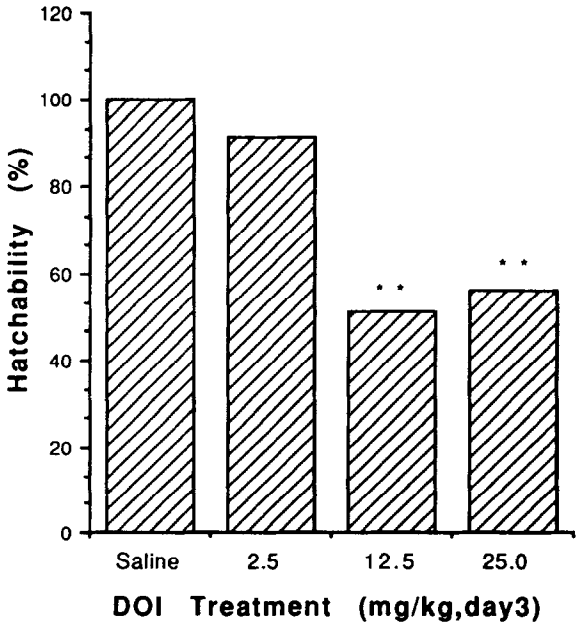

FIG. 1. DOI supresses hatchability dose relatedly when injected into eggs on day 3 of incubation. No significant effect of DOI upon viability was observed on days 6 or 13. Relative to saline-injected controls, fewer chicks hatched from eggs injected with 12.5 mg or 25 mg DOI/kg egg. $^{**}p < 0.01$; DOI vs. saline.

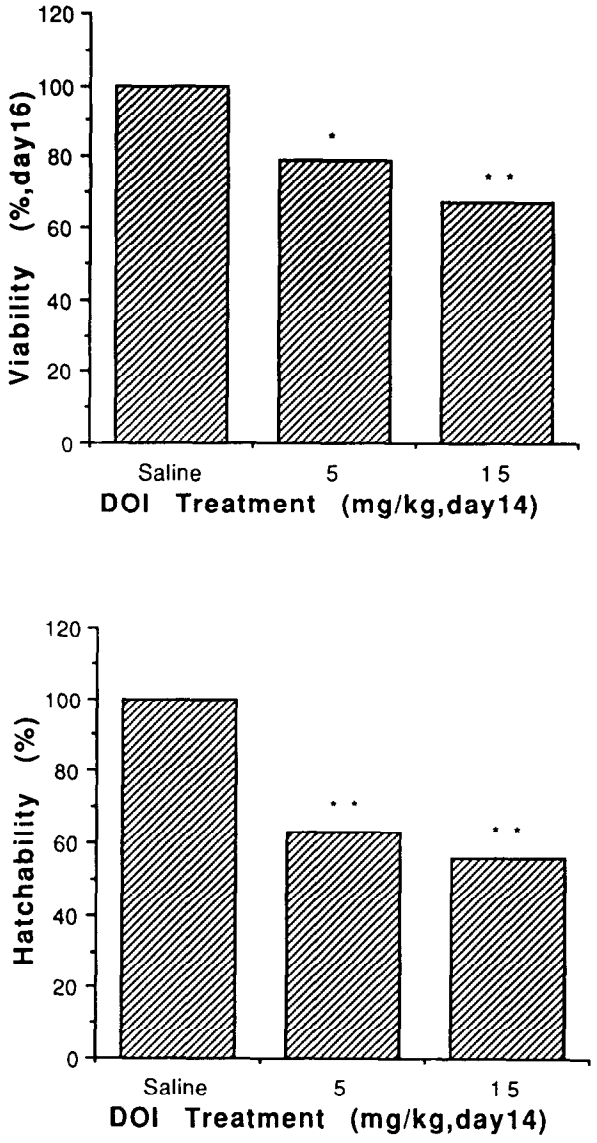

FIG. 2. DOI suppressed hatchability at a lower dose (i.e., 5 mg/kg) when injected on day 14 of incubation compared with higher doses needed for reduced hatchability when injected on day 3 (see Fig. 1). Furthermore, significant reductions in viability occurred by day 16 (see text), within 2 days of injecting DOI, which was not observed during the 10 days after injection of any dose of DOI on day 3. $*p < 0.05$, $**p < 0.01$; DOI vs. saline.

anomaly in DOI-exposed hatchlings and to determine if the lethal and dysmorphogenic effect of DOI could be induced by injection later during development (e.g., on day 18), if embryonic behavior is altered within less than a day after injection of DOI, and if the 5-HT$_2$ antagonist RIT is effective against these toxic actions of the agonist.

EXPERIMENT 3A. INJECTION OF 5 mg DOI/kg OF EGG, WITH OR WITHOUT RIT, ON DAY 18 OF EMBRYOGENESIS: EFFECTS UPON HATCHABILITY AND INDUCTION OF HERNIATED UMBILICI

This experiment was performed to determine if 5 mg DOI/kg of egg injected on day 18 of embryogenesis was apparently more toxic, as it relates to hatchability, compared with the same dose injected on day 14 of embryogenesis. We also wanted to determine if it will induce herniated umbilici when injected this late during embryogenesis. Moreover, because we were postulating a selective 5-HT$_2$ mechanism for DOI's toxicity, we administered the antagonist RIT before DOI in an attempt to block or attenuate one or both effects of the agonist. Preliminary experiments indicated that doses of up to 1 mg RIT/kg of egg injected on the morning of the 18th day of embryogenesis had no discernable effect upon any parameters we used to assess DOI's toxicity. In this experiment 14–16 eggs with viable embryos were injected with the tartaric acid vehicle for RIT or RIT itself, followed 3 h later with DOI. Because we had not observed any toxic effects of RIT by itself, at higher doses, we did not include separate RIT groups. The three drug groups consisted of a group injected with DOI and two groups injected with either 0.2 mg RIT/kg or 0.4 mg RIT/kg, at 0900, followed 3 h later by a dose of 5 mg DOI/kg.

Results and Discussion

Injection of this dose of DOI caused about an 80% reduction in hatchability (i.e., 3 out of 14 hatched) compared with about a 40% reduction in hatchability when the same dose of DOI was injected on day 14 of embryogenesis, supporting our hypothesis that the older embryo would again show greater sensitivity to the toxic effects of the 5-HT$_2$ agonist. Chi-square analyses of the hatchability data for all four groups indicated an overall significant effect, $\chi^2(3) = 22.13$, $p = 0.0001$. Pretreatment of the eggs with RIT resulted in a dose-related antagonism of the lethal effects of DOI. Although about 70% (11 out of 16) of the DOI + 0.2 mg RIT/kg group hatched, this still represented a significant reduction, relative to the 100% of chicks hatching in the control group, $\chi^2(1) = 5.59$, $p = 0.018$. However, the high dose of RIT (0.4 mg/kg) allowed 80% (12 out of 15) of the chicks also injected with DOI to hatch. Thus, there was no significant difference between the control group and the high dose RIT group, upon this measure of toxicity (Fig. 3).

The incidence of herniated umbilici was 100% (3 out of 3)

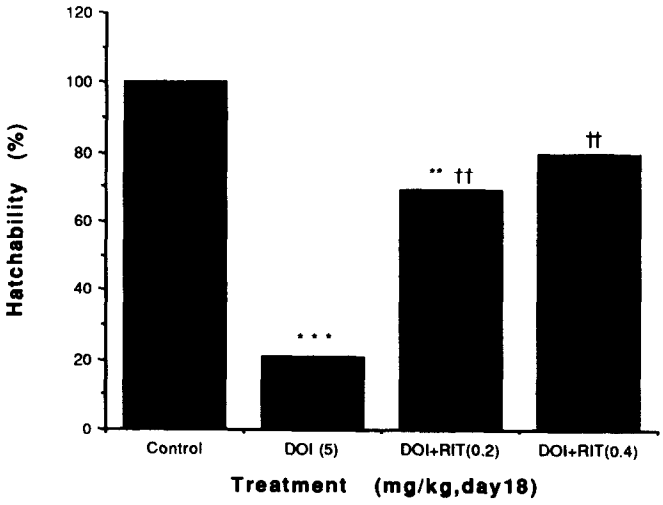

FIG. 3. Suppression of hatchability by 5 mg DOI/kg injected on day 18 of incubation was not completely blocked by 0.2 mg RIT/kg injected 3 h earlier. The higher dose of RIT (0.4 mg/kg) did so. $***p < 0.001$, DOI vs. tartrate/saline controls; $**p < 0.02$, DOI + RIT (0.2 mg/kg) vs. controls; $\dagger\dagger p < 0.01$, vs. DOI.

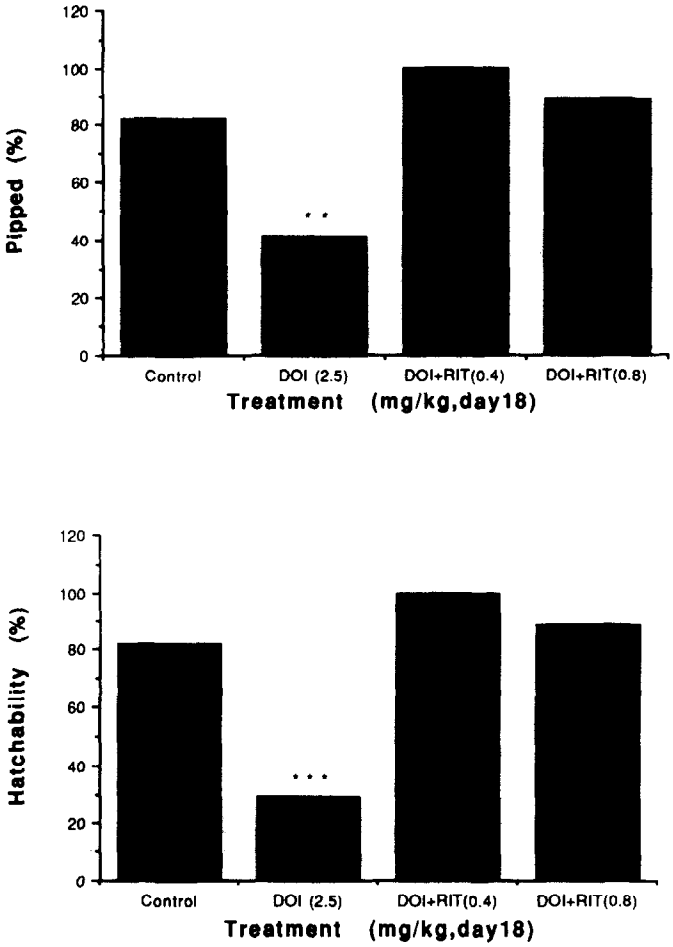

FIG. 4. A significant reduction in pipping the eggshell prior to hatching was caused by injection of 2.5 mg DOI/kg on day 18 of incubation. While all embryos that pipped their shells in the control and DOI + RIT groups eventually hatched, only 5 out of 17 of the pipped eggs in the DOI group had hatchlings emerge from them. $^{**}p < 0.02$; $^{***}p < 0.001$, DOI vs. all other groups.

in the few chicks hatching in the 5 mg DOI/kg group, while 75% (9 out of 12) and 82% (9 out of 11) of the chicks hatching in the high and low dose RIT groups, respectively, also had herniated umbilici. Chi-square analyses indicated that RIT, at either dose, did not reduce the incidence of this anomaly. The mean size of the herniated umbilici ranged from about 4 to 6 mm. As for the incidence of herniations, RIT did not afford any significant protection against the effect of this dose of DOI upon this parameter. However, the body weights of the hatchlings where not affected by treatment with 5 mg DOI/kg or DOI plus either dose of RIT (data not shown).

Thus, it appears that the capacity of DOI to induce herniated umbilici exceeds its capacity to prevent chicks from hatching when injected late during embryogenesis. Alternatively, the mechanism whereby DOI causes the herniated umbilici is not related to its action at 5-HT_2 receptors; hence, the inability of RIT to block or attenuate this effect of DOI. It is also possible that the DOI injection prevented the most affected embryos from ultimately hatching, leading to the relatively few hatchlings with which to compare the efficacy of pretreatment with RIT vis-à-vis the incidence or size of herniated umbilici. To test these possibilities, the above experiment was repeated, except that half the dose of DOI was injected and the doses of RIT were doubled.

EXPERIMENT 3B. INJECTION OF 2.5 mg DOI/kg OF EGG, WITH OR WITHOUT RIT, ON DAY 18 OF EMBRYOGENESIS: EFFECTS UPON HATCHABILITY AND HERNIATED UMBILICI

When 2.5 mg DOI/kg of egg was injected on day 18 of embryogenesis, about 40% (7 out of 17) of the embryos pipped their shells, compared with about 80% (14 out of 17) in the control group and 100% (18 out of 18), and 90% (16 out of 18) in the low and high dose RIT groups, respectively, $\chi^2(3) = 20.31$, $p = 0.0001$. While all chicks in the control and RIT pretreated groups that pipped their shells eventually hatched, 5 of 17 (30%) in the 2.5 mg DOI/kg group hatched, $\chi^2(3) = 27.70$, $p = 0.0001$, compared with 20% having hatched after 5 mg DOI/kg was injected on the same day in the previous experiment. This is a conservative estimate of the hatchability in the DOI group in this experiment, as only 82% of the control group hatched. Moreover, the injection of either 0.4 or 0.8 mg RIT/kg totally blocked the lethal effect of this dose of DOI, allowing 100 and 89% hatchability, respectively (Fig. 4).

Unlike the previous experiment, while 4 out of 5 (80%) of the DOI chicks had herniated umbilici, the 5-HT_2 antagonist also reduced the incidence of this anomaly in a dose-related manner. Of the 18 chicks hatching in the low dose RIT group, 11 (61%) had herniated umbilici, while only 4 out of 16 (25%) in the high dose RIT group had (small) herniated umbilici. ANOVA of the sizes of this anomaly, revealed a significant overall treatment effect, $F(3, 49) = 12.10$, $p = 0.0001$. Subsequent contrasts revealed that both RIT groups had significantly smaller herniated umbilici, compared with the DOI group, although hatchlings in the low RIT dose group still had

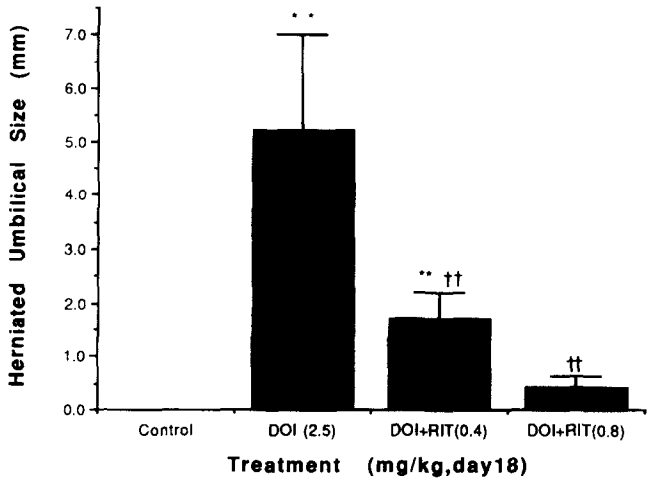

FIG. 5. Herniated umbilici were significantly greater in the chicks that hatched from eggs injected with 2.5 mg DOI/kg on day 18 of incubation, compared with the Controls and both groups injected with DOI + RIT. The low dose of RIT caused a significant reduction in the size of the umbilici herniated by DOI, but they were still significantly larger than the Controls and the barely detectable herniations in the group injected with DOI and the higher dose of RIT. The high dose of RIT completely blocked the DOI-induced herniations, as defined. The vertical line above the histograms represent + 1 SEM. $^{**}p < 0.01$ vs. Controls; $\dagger\dagger p < 0.01$ vs. DOI 2.5 mg/kg; $\#p < 0.05$ vs. DOI + RIT (0.4 mg/kg).

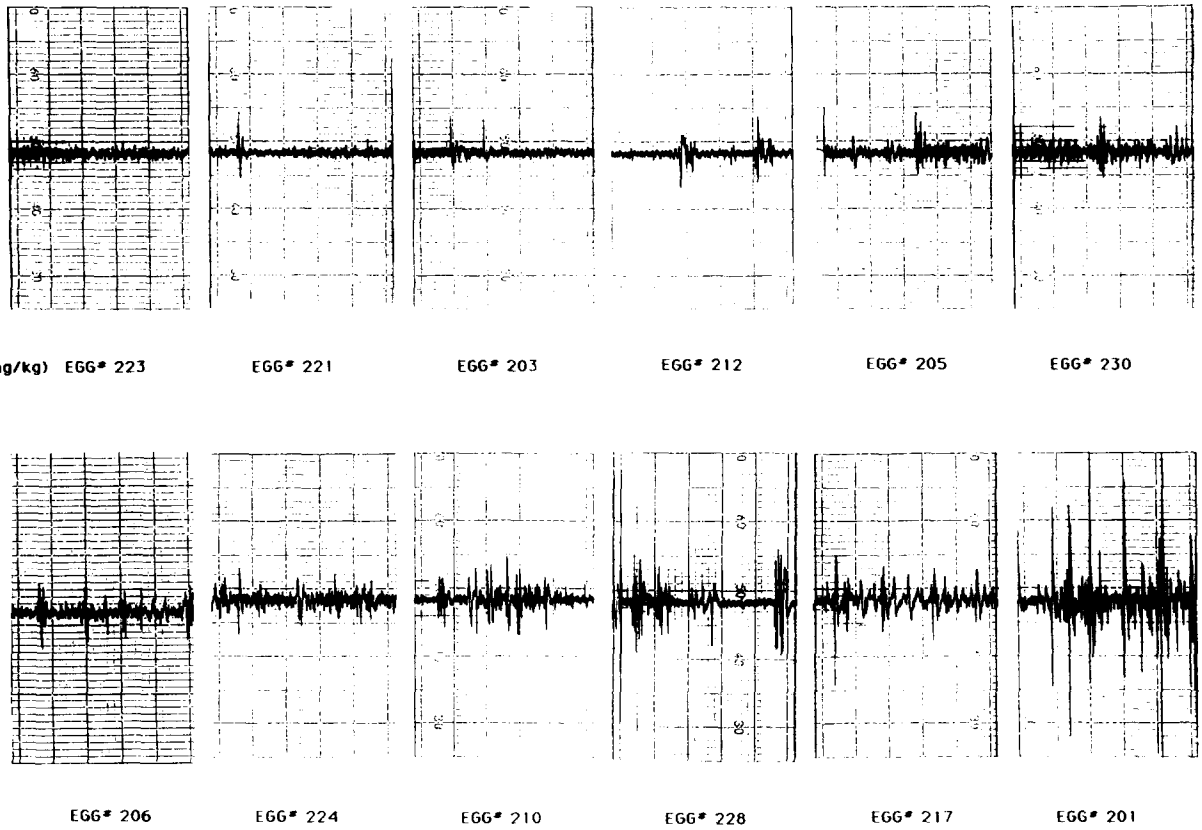

FIG. 6. Analog recordings of embryonic motility on day 19 of incubation after injection of saline or DOI (2.5 mg/kg) in the afternoon of the previous day. Records represent about 30 s midway through the recording period. One of the six DOI-exposed embryos showed virtually no evidence of motility at this time. Records are arranged from left to right showing apparently least to greatest activity in both groups.

herniated umbilici that were significantly enlarged, relative to controls (Fig 5). As in the previous experiment in which a higher dose of DOI and a lower dose of RIT were used, there were no differences in body weights of hatchlings exposed to DOI or the combination of drugs (data not shown).

EXPERIMENT 4. INJECTION OF 2.5 mg DOI/kg OF EGG ON DAY 18 UPON MOTILITY OF EMBRYOS ON DAY 19 OF EMBRYOGENESIS

Figure 6 shows portions (i.e., about 30 s) of the analog records of spontaneous embryonic motility from each of six embryos in eggs injected with either avian saline or DOI (2.5 mg/kg egg) the evening of day 18 of embryogenesis and monitored on day 19. Visual inspection should be sufficient to conclude that the 5-HT$_2$ agonist produced profound effects upon spontaneous motility 1-2 days before the embryos were scheduled to hatch. The objective measures, which seemed best for quantification of embryonic motility with the A/D converter and Superscope, are depicted in Fig. 7. Because each egg (embryo) had slightly different offset voltages when the electrodes were inserted, we chose to use the standard deviation, in addition to the maximum and minimum voltages generated by movement of embryos within the egg. We also used the standard deviation as a measure of variability and total power resulting from the embryos' movements. Although power is usually measured as Root Mean Square (RMS), the standard deviation is very highly correlated with RMS (unpublished observations) and it allowed us to analyze the data without subtracting the individual offset voltages. These measures were, as predicted from the analog recordings, dramatically reduced after DOI. Thus, it appears that this automated and objective method of monitoring gross behavior of chick embryos should be sensitive to even more subtle changes than those produced by this high dose of DOI.

GENERAL DISCUSSION

We have presented data that strongly suggest there are important functional and/or toxicological consequences of excessively stimulating 5-HT$_2$ receptors during late development of the chick embryo. As a consequence of the suppression of embryonic motility late during development, the selective agonist DOI interfered with one or more components of the complex repertoire responsible for hatching. The fact that pipping didn't seem to be or was moderately affected, depending upon the dose and time of administration, suggests that more complex, perhaps spinally and/or supraspinally mediated coordinated behaviors, other than pipping, are involved to a greater extent or are more sensitive to DOI's disruptive action. Because 5-HT containing pathways have been reported not to be approaching full morphological development in the CNS until early into the third trimester of development or later in this species (26,30,31), it should not be surprising that we observed greater resistance to the toxic effects of DOI (upon hatchability) when we injected the agonist early during development (e.g., higher doses were needed when injected early, compared with later during development). The young chick

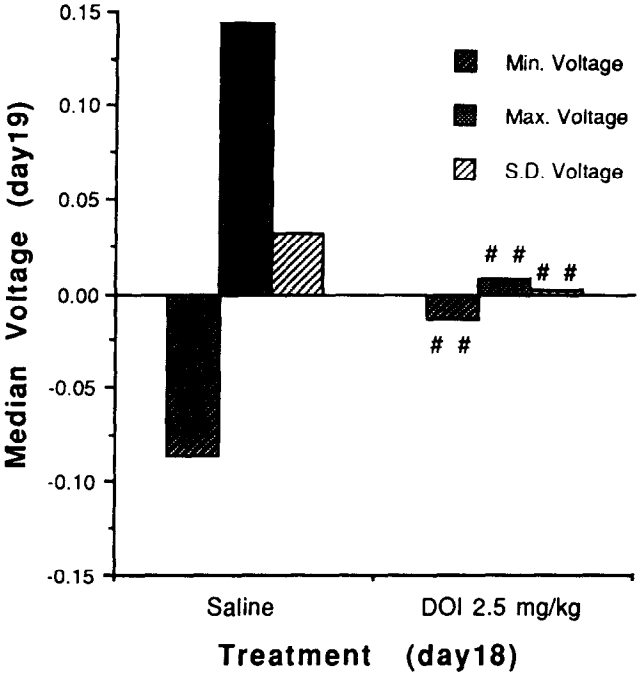

FIG. 7. Histogram plots of three parameters that showed significant suppression of motility on day 19 of incubation after injection of 2.5 mg DOI/kg the night before. They represent the median values for averaged minimum, maximum, and standard deviations of voltages monitored during 40 sweeps lasting 5 s each after allowing the embryos 3 min of acclimation subsequent to insertion of electrodes 180° apart, midway between the two poles of the eggs, as they lay horizontally. #$p < 0.004$; Mann-Whitney test.

embryo is not generally resistant to the toxic effects of drugs and chemicals; their individual toxicological profiles may depend upon the presence of functional receptors when their action is mediated pharmacodynamically through such selective membrane-bound lipoproteins. For example, opioid receptors, or at least binding sites for opioids, have been reported to be present very early during embryonic development of this species (4). Moreover, the expression of toxicity was manifest as reduced viability shortly after injection of an opioid on day 3 of incubation (13) or as inhibited embryonic motility in the young embryo (22).

We also observed evidence of herniated umbilici in hatchlings from eggs, regardless of whether they were injected early or late in development, indicating that the DOI injected earlier must have survived mixed function metabolic inactivation processes, which in the chick embryo are not very active or inducible until the middevelopmental period (18). Thus, the vascular, or other insults responsible for this structural anomaly probably occurred late in development, regardless of the stage of development when DOI was injected into the egg.

The probability that DOI's actions upon hatchability and the induction of the herniated umbilici was via a selective action on 5-HT_2 receptors is strongly supported by the efficacy of RIT to block or signigicantly attenuate these effects. Furthermore, we have observed what appears to be a downregulation of 5-HT_2 receptors (i.e., [^{3}H]-ketanserin binding sites) in chick embryo brain on day 18 of embryogenesis, after injection of RIT on day 17 of development(Kim and Sparber, submitted), again strongly supporting the notion that this subclass of serotonin receptors is functional and pharmacologically responsive in the direction predictable from studies with mature subjects of other species (17,32). While we have offered evidence that there are 5-HT_2 receptors that show functionality during later stages of chicken embryonic development, we have examined only a few, acute manifestations of their manipulation with a selective agonist and antagonist. A fuller characterization of their appearance and responsiveness during different stages of development will be necessary for determining the profile of this receptor's functional significance in normal developmental processes and/or their perturbation during various stages of development upon more subtle and/or longer lasting effects upon neurochemistry and behavior, beyond the perinatal period of this species.

We also may have inadvertently stumbled upon a model for studying the etiological factors responsible for a high incidence of spontaneous abortions in pregnant women who had elevated 5-HIAA levels in their urine and have responded positively to treatment with the less-specific drug cyproheptadine (29), which has 5-HT_2 antagonist properties, among others. The possibility that RIT-like drugs would be efficacious in this regard should also be entertained. The data also suggest that RIT-like drugs may be safe and efficacious against drug-associated life-threatening or more subtle manifestations in immature mammals undergoing severe opioid withdrawal or exposed to acutely toxic doses of cocaine, both of which appear to result, at least in part, from overactivity at 5-HT_2 receptors. We are currently studying these possibilities, as well as the relationship between doses of RIT that show efficacy and those that may be toxic or functionally teratogenic.

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