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2,4,5-Trichlorophenoxyacetic Acid Causes Behavioral Effects in Chickens at Environmentally Relevant Doses

Abstract. *Administration of the herbicide 2,4,5-trichlorophenoxyacetic acid to incubating chicken eggs alters behavior after hatching. Single doses, with no morphological effects, retard learning (lowest dose, 7 milligrams per kilogram of body weight) and increase general activity (27 milligrams per kilogram) and jumping (13 milligrams per kilogram). Day 15 of incubation is the most susceptible stage of development.*

As far as we know, there has been only one earlier study reporting teratological effects of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) on behavior (*1*). Administration of 2,4,5-T (100 mg/kg) to pregnant rats produced no morphological deformities in the offspring, but it did cause them to have learning deficits and to exhibit increased activity in the open field. We have shown that even lower doses can produce behavioral abnormalities in the chicken. A single dose of 2,4,5-T [Sigma; containing 0.03 part per million of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), according to the Australian Government Analytical Laboratory] was administered to the chicken egg on either day 8 or day 15 of incubation (*2*). A third group received 2,4,5-T by intraperitoneal injection on day 2 after hatching. The 2,4,5-T was suspended in a 0.5 percent solution of gum tragacanth made with sterilized, distilled water. Control animals (matched for each separate hatching) received the vehicle alone. Doses were calculated according to the weight of the chicken or egg at the time of injection and ranged from 7 to 53 mg/kg for eggs and 75 to 225 mg/kg for chicks after hatching.

The median lethal dose (LD_{50}) on day 2 after hatching was 200 mg/kg, and on day 15 of incubation it was 53 mg/kg. There was 70 percent hatching in eggs given 2,4,5-T on day 15 of incubation at doses up to 27 mg/kg. Some 5 to 10 percent of the hatched chicks, irrespective of dose, showed abnormal leg development; they either dragged one leg or held it off the ground. Approximately 5 percent of the treated chicks showed depigmentation of feathers and down. Both of these effects have been reported (*3*). No differences in body weight gain were observed in chicks receiving 2,4,5-T during incubation or at doses less than 150 mg/kg on day 2 after hatching.

The chicks without apparent morphological deformities (after doses shown in Fig. 1) were given behavioral tests in week 2 after hatching. The tester was unaware of the treatment that each chick had received. There were 8 to 20 chicks in each group tested. We measured the activity in an unfamiliar environment on day 7 of life by placing the chick in a gray-walled box (30 cm by 30 cm with 25-cm walls). Cumulative activity for a 3-minute interval ("general activity") was scored by an Animex activity meter, and the number of jumps made was counted by eye. We scored ambulation for some groups by dividing the floor of the box into quadrants and counting the number of times the chick crossed into a new quadrant. We tested visual discrimination learning on day 9 of life by using the "pebble floor" test (*4*), which consists of a search for grains of food scattered over a background of small pebbles adhering to the floor. Within a 60-peck trial, a measure of visual learning is generated by recording the number of incorrect responses (pecks at pebbles) within consecutive 20-peck intervals.

General activity, jumping, and visual learning rate were altered by 2,4,5-T treatment (Fig. 1). Since the same doses were administered on day 8 and day 15 of incubation, these data could be examined by a two-way analysis of variance (that is, dose, age, and their interaction). A one-way analysis of variance was used for the data from day 2 after hatching. General activity scores conformed to a parametric distribution, but jumping and learning scores required logarithmic transformation.

Jumping behavior proved to be the most sensitive parameter affected by 2,4,5-T. For those treated on days 8 and 15 of incubation, jumping showed both a significant dose effect ($F = 2.78$; d.f. = 3, 90; $P < .05$) and significant interaction between age and dose ($F = 3.33$; d.f. = 3, 90; $P < .05$). Comparisons of the groups given the two highest doses with their control groups (t-test) showed a significant increase in jumping in the groups treated with 13 mg/kg ($P < .05$) and 27 mg/kg ($P < .01$) on day 15 of incubation, but there were no significant differences in those treated on day 8 (see Fig. 1). There was also a significant dose effect for jumping in the groups treated on day 2 after hatching ($F = 4.06$; d.f. = 2, 20; $P < .05$), and a t-test of data for the control group and the group receiving a dose of 150 mg/kg revealed a significant increase in jumping after 2,4,5-T treatment ($P < .025$), this dose being tenfold higher than the lowest dose found to be effective on day 15 of incubation.

General activity, which includes measures of jumping, ambulation, and other movements, shows a similar tendency to increase after 2,4,5-T treatment on day 15 of incubation and day 2 after hatching (Fig. 1). Analyses of variance just failed to reveal significant effects, but t-tests of data for the control groups and those groups that had received the highest doses at each age revealed a significant elevation of general activity in the groups treated with 27 mg/kg on day 15

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of incubation ($P < .01$) and those receiving 150 mg/kg on day 2 after hatching ($P < .025$). There were no significant effects after treatment on day 8.

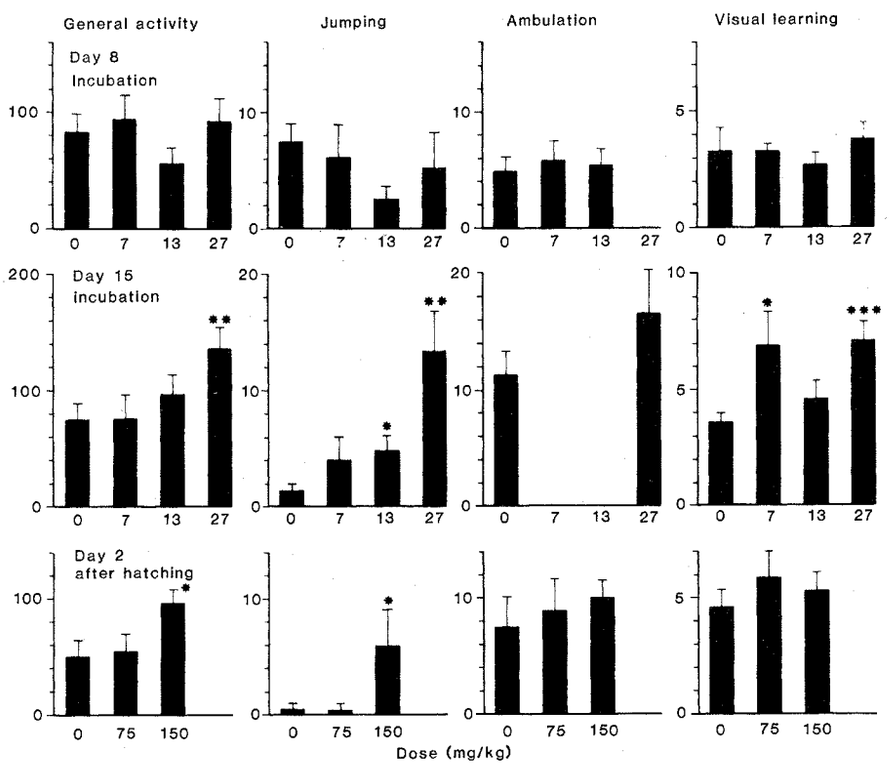
Ambulation, the other component of general activity that we measured, did not show any dependence on dose and no significant differences were found as a result of examination of the data by *t*-tests, even though there was a tendency for ambulation to increase with increasing dose after treatment on day 15 of incubation and day 2 after hatching (Fig. 1). Thus, as found in rats, 2,4,5-T has long-lasting effects on activity, but in chicks the most sensitive parameter contributing to the increased activity is jumping rather than ambulation, which may indicate increased fear responsiveness to novelty.

There was a significant dose effect for visual discrimination learning in those groups treated on days 8 and 15 of incubation ($F = 4.19$; d.f. = 3, 86; $.005 < P < .01$) but not for those treated on day 2 after hatching. Comparison of treated animals with their controls at each age revealed a significant slowing of learning in those treated on day 15 of incubation with 27 mg/kg ($P < .001$) and 7 mg/kg ($.01 < P < .025$), but no significant effects were present after treatment at the other two ages.

Day 15 of incubation appears to be a stage of maximum sensitivity to the behavioral effects of 2,4,5-T. Neuronal cell division is at a peak on day 8 of incubation and is largely completed by day 15 when synaptic proliferation is occurring (*5*). Synaptic proliferation is completed before day 2 after hatching (*4*). The brain therefore seems to be most sensitive to 2,4,5-T at a time of maximum synaptic formation. The fact that 2,4,5-T caused adverse effects at lower doses than reported previously may indicate an extreme susceptibility of the developing nervous system and hence behavior. This is consistent with one report of the possible adverse effects of 2,4,5-T in the human species (*6*).

The extent to which our results are due to 2,4,5-T or to very small amounts of its highly toxic contaminant, TCDD, remains unresolved. However, it is important to remember that in this study we used 2,4,5-T of greater purity, particularly with respect to TCDD content, than that available commercially and sprayed in the environment. Based on the concentration recommended in Australia for spraying blackberries (*7*), which is 2.8 liter/ha of commercial 80 percent 2,4,5-T (weight to volume), 1 m^2 would receive 224 mg of 2,4,5-T. Since the surface area of an egg is approximately 50 cm^2, the dose of 2,4,5-T received by one egg after spraying would be 1.1 mg or 22 mg/kg, which is greater than the lowest dose found to be behaviorally teratogenic. As 2,4,5-T has been found to pass through the egg shell (*8*), it may therefore present a real risk to bird species. It is questionable whether there is a dose below which 2,4,5-T can be said to have no effect since some individuals appear to be highly susceptible. At all the doses we used, morphological deformations were seen in some of the treated chicks, and, as the raw data for jumping in Fig. 2 illustrate, some individuals with no morphological deformations were highly susceptible to the behavioral effects of 2,4,5-T, even at the lowest dose (7 mg/kg) that we administered.

There is considerable species variation in sensitivity to the toxic effects of 2,4,5-T. At present one of the best indicators of interspecies toxicity is the rate at which 2,4,5-T is metabolized and excreted (*9*). Based on such measures, chicks and rats appear to be of roughly similar sensitivity whereas humans have



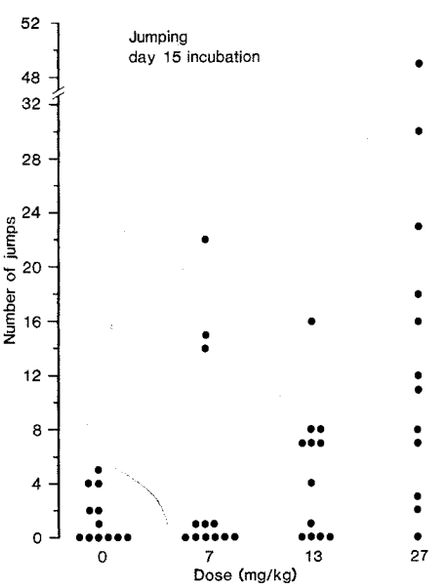


Fig. 1 (left). Plots of means and standard errors for four behavioral measures after the administration of 2,4,5-T at the doses indicated on day 8 or 15 of incubation or day 2 after hatching. Behavior has been tested in week 2 of life. The ordinate scales are as follows: for general activity, counts on the electronic activity meter in the total 3 minutes of scoring; for jumping, the number of jumps in 3 minutes; for ambulation, the number of times the chick crossed with both feet into a new quadrant of the box; and for visual learning, the number of errors (pecks at pebbles) in the last 20 pecks of the pebble-floor learning task. In the latter test, a higher error score indicates slower learning. The asterisks indicate significant differences from the control groups as determined by one-tailed *t*-tests: *, $P < .05$; **, $P < .01$; and ***, $P < .001$. Fig. 2 (right). The raw data for jumping scores obtained during 3 minutes in a novel environment after treatment with 2,4,5-T on day 15 of incubation. Each point represents the score for a single individual. The fact that some individuals are highly susceptible to the behavioral teratological effects of 2,4,5-T is demonstrated by the finding that, even after treatment with the lowest dose (7 mg/kg), some individuals show a marked increase in jumping.

at least a threefold greater sensitivity (*10*). Our findings therefore imply a risk to the human species.

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Neurosecretory Granules: Evidence for an Aging Process Within the Neurohypophysis

Abstract. *When cysteine labeled with sulfur-35 is injected into the third ventricle of the rat brain, it is first incorporated into only one of the two populations of neurosecretory granules that can be isolated on an isosmotic gradient. The second population of granules is labeled much later. Stimulation of hormone release from isolated labeled neural lobes and subsequent isolation of neurosecretory granules at different times after the injection of labeled cysteine shows that the radioactivity decreases in only one population of granules. One of the fractions of the gradient represents the granules found near the release site; the second population is probably located deeper in the nerve endings or in the nerve swellings. Whereas neurophysins are found in both populations, smaller proteins can only be detected in one. Thus it appears that neurosecretory granules undergo an aging process and that isosmotic gradients can separate the aged granules from those newly formed.*

Neurosecretory granules (NSG) in the hypothalamo-neurohypophysial tract are formed in the magnocellular neuron of the supraoptic and paraventricular nuclei (*1*). Labeling with [^{35}S]cysteine indicates that the magnocellular neurons mainly contain a large precursor protein (~ 20,000 daltons), which presumably is packaged into granules. The contents of the granules mature during transport toward the neurosecretory nerve terminals. When the granules reach the neural lobe, almost all the precursor has been converted into proteins of 12,000 ± 2,000 daltons, neurophysins, and peptides that may include the hormones oxytocin and facopressin (*2*).

The neurosecretory axons in the neurohypophysis can be subdivided into three compartments, all of which contain NSG (*3*): undilated axons containing few NSG, nerve endings characterized by the presence of microvesicles, and nerve swellings containing secretory granules but few if any microvesicles. We previously showed that the mean diameter of NSG in the endings differs from that of NSG in the swellings (*4*). Furthermore, we showed that fractionation of NSG from the neural lobe of rats on an isosmotic gradient yields two populations of NSG, each of which contains neurophysins, oxytocin, and vasopressin. One population has an isopycnic density of 1.13 g/cm^3 and is insensitive to osmotic changes in the surrounding medium, whereas the other sediments at a density of 1.11 g/cm^3 and is much affected by the osmotic pressure of the surrounding medium. Furthermore, the mean diameter of osmotically insensitive NSG after fixation is very similar to that of the NSG in the nerve endings, whereas the mean diameter of the osmotically sensitive NSG resembles that of the granules in the swellings.

In this report we present evidence that the osmotically insensitive granules are newly formed NSG (NF-NSG) that have recently arrived at the neural lobe and that the osmotically sensitive NSG are probably derived from the former. Furthermore, we show that the NF-NSG are located in the nerve endings, whereas most of the aged granules (A-NSG) cannot immediately release their contents and are thus likely to be found in the swellings. Protein species of a molecular weight lower than that of neurophysins can be demonstrated in the A-NSG. This suggests possible postmaturational cleavage of intragranular proteins.

Albino (Wistar) male rats weighing 250 to 300 g were anesthetized with ketamine chlorohydrate (10 mg per 100 g of body weight) and given intracisternal injections of [^{35}S]cysteine that was obtained from the supplier (New England Nuclear, 500 Ci/mmole) or by reduction of [^{35}S]cystine (470 Ci/mmole) with 10 m*M* dithiothreitol in 0.1*M* phosphate buffer (*p*H 7.0). Each rat was injected with 10 μl of the final solution (50 μCi of radioactive cysteine). The rats were decapitated at different times after the injection and their neural lobes were dissected out in less than 1 minute. Immediately after their isolation, ten of the radioactive lobes were homogenized in 1.0 ml of 0.3*M* sucrose buffered at *p*H 6.8 with 10 m*M* Hepes, and mixed with 20 nonradioactive neural lobes homogenized under the same conditions. The NSG were isolated as described by Nordmann *et al.* (*4*). The gradient fractions were collected with a Buchler device, and a portion of each fraction was kept for a determination of density. Scintillation fluid was added to the fractions and their total radioactivity was measured with a

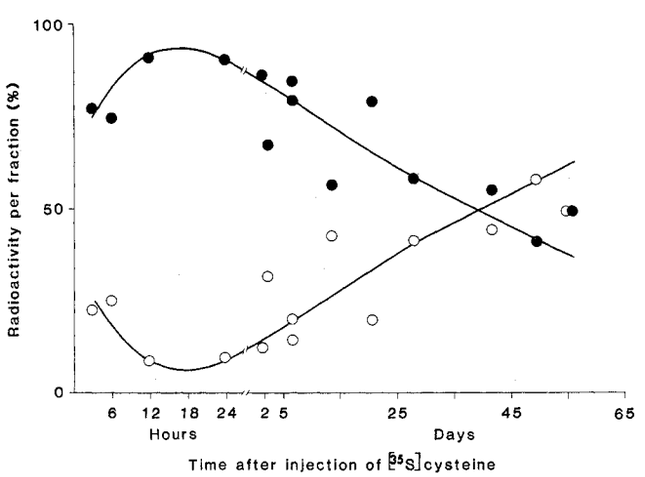

Fig. 1. Time course of the appearance of [^{35}S]cysteine in NSG fractions isolated on a sucrose-metrizamide isosmotic gradient at different times after the injection of the isotope. The radioactivity in NF-NSG (●) and A-NSG (○) was measured and the results are given as the percentage of the total radioactivity found in both fractions.

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