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Effect of 2,4-dichlorophenoxyacetic acid on rat maternal behavior

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Abstract

Exposure to 2,4-dichlorophenoxyacetic acid (2,4-D) has several deleterious effects on the nervous system such as alterations in the concentrations of neurotransmitters in the brain and/or behavioral changes, myelination rate, ganglioside pattern [Bortolozzi, A., Duffard, R., Antonelli, M., Evangelista de Duffard, A.M., 2002. Increased sensitivity in dopamine D(2)-like brain receptors from 2,4-dichlorophenoxyacetic acid (2,4-D)-exposed and amphetamine-challenged rats. Ann. N.Y. Acad. Sci. 965, 314-323; Duffard, R., García, G., Rosso, S., Bortolozzi, A., Madariaga, M., DiPaolo, O., Evangelista de Duffard, A.M., 1996. Central nervous system myelin deficit in rats exposed to 2,4-dichlorophenoxyacetic acid throughout lactation. Neurotoxicol. Teratol. 18, 691–696; Evangelista de Duffard, A.M., Orta, C., Duffard, R., 1990. Behavioral changes in rats fed a diet containing 2,4-dichlorophenoxyacetic butyl ester. Neurotoxicology 11, 563–572; Evangelista de Duffard, A.M., Bortolozzi, A., Duffard, R.O., 1995. Altered behavioral responses in 2,4-dichlorophenoxyacetic acid treated and amphetamine challenged rats. Neurotoxicology 16, 479–488; Munro, I.C., Carlo, G.L., Orr, J.C., Sund, K., Wilson, R.M. Kennepohl, E. Lynch, B., Jablinske, M., Lee, N., 1992. A comprehensive, integrated review and evaluation of the scientific evidence relating to the safety of the herbicide 2,4-D. J. Am. Coll. Toxicol. 11, 559–664; Rosso et al., 2000], and its administration to pregnant and lactating rats adversely affects litter growth and milk quality. Since normal growth of the offspring depends on adequate maternal nursing and care, we evaluated the effect of 2,4-D on rat maternal behavior as well as the dam's monoamine levels in arcuate nucleus (AcN) and serum prolactin (PRL) levels. Wistar dams were exposed to the herbicide through the food from post partum day (PPD) 1 to PPD 7. Dams were fed either with a 2,4-D treated diet (15, 25 or 50 mg 2,4-D/kg/day bw) or with a control diet. We observed that maternal nesting behavior was not modified by 2,4-D treatment. However, mother–pup interactions, specially the nursing behavior, were altered. Retrieval, crouching and licking of pups were reduced or suspended after 2,4-D treatment. We also observed an increase in the latency of retrieval and crouching in the dams treated with the herbicide. Dams showed movement along cage peripheries, food consumption during the light phase and high self-grooming. In addition of the deficits observed in maternal behavior parameters, increased catecholamine levels and a drastic decrease in indolamine levels in the AcN of treated dams were determined. Serum PRL levels were also diminished by 62%, 68% and 70% with respect to control dams in the 15, 25 and 50 mg 2,4-D/kg bw treated dams, respectively. In conclusion, exposure to 2,4-D during the first post partum days produced changes in maternal behavior, serum prolactin and monoamine levels in the AcN of treated dams.

Keywords: Maternal behavior; 2,4-dichlorophenoxyacetic acid; Prolactin; Neuroendocrine disruption; Lactation

1. Introduction

A large amount of data indicates that substances naturally present in the environment or of anthropogenic origin are able to interfere with the functioning of the endocrine system in vertebrates (Crews et al., 2000; Taylor and Harrison, 1999). Such interference can range from a disruptive to a modulator effect, depending on the mode of action of the substance, its concentration in the organism and, primarily, the timing of exposure. Behavioral indices may be particularly sensitive to perturbation of hormonal systems because they represent the endpoint of integrated systems, and in particular, behavior is a sensitive and broad indicator of disturbances in the central nervous system (CNS) (Suomi, 1997).

There are very few studies on developmental toxicology that focus on the xenobiotic's effects on maternal care. Maternal behavior can be defined as the activities that the mother performs

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oriented to care for the pups, and that lead to the normal development and survival of her offspring, providing the newborn with protection, warmth and nutrition. In the rat, the components of maternal behavior include nest building, retrieval of the pups into the nest and grouping them together, crouching over the young to facilitate suckling and to provide warmth, licking the young to stimulate micturition and defecation, and defense of the young from intruders. These behaviors appear to be coordinated by an integrated neural program (Numan, 1994; Rosenblatt et al., 1985). Alterations of maternal behavior have been described after exposure to different xenobiotics. Studies in rats have shown that dams treated with 30 mg/kg of cocaine chronically throughout gestation delayed the onset of maternal behaviors (i.e., crouching, nestbuilding, and retrieval) (Johns et al., 1994, 1997). Exposure to 10 μg/kg/day of Bisphenol A during development or during pregnancy decreased maternal behavior (Palanza et al., 2002a,b). 3,4,3′,4′-tetrachlorobiphenyl (PCB 77) administered during gestation reduced the amount of nursing time and increased the amount of maternal licking and grooming of the litters (Simmons et al., 2005).

The hormonal changes accompanying pregnancy and parturition play a critical role in preparing the female to respond maternally toward her newborn young (Bridges et al., 1996). Roles for steroid hormones, estradiol and progesterone, as well as oxytocin, cholecystokinin and prolactin (PRL) have been demonstrated to be critical in this behavioral process.

In particular, PRL plays an important role in the initiation and maintenance of lactation. Apart from its well-known effects upon the mammary gland regulating the production and composition of milk, within the brain, PRL plays a significant role in the induction and regulation of all the components of maternal behavior (Bridges et al., 1985, 1990).

In addition, several neurotransmitters have been shown to participate in the induction and maintenance of maternal behavior. Rat dams that were given ventral tegmental area microinfusions of 6-hydroxydopamine (dopamine-depleting agent) during lactation showed a persistent deficit in pup retrieval (Hansen et al., 1991; Hansen, 1994). The infusion of a dopamine receptor antagonist into the nucleus accumbens inhibits retrieval and licking components of maternal behavior. These studies suggest that expression of the behavior requires dopamine (Keer and Stern, 1999). On the other hand, increased dopaminergic tone in the TIDA system inhibits maternal behavior indirectly, through decreased PRL secretion (Bridges et al., 1990; González-Mariscal et al., 2004). Also, dopaminergic stimulation of some components of maternal behavior, such as retrieval and licking of pups, are mediated by activation of D1 receptors, while D2 receptors have an inhibitory effect, mainly on nursing behavior (Miller and Lonstein, 2005).

The widespread use of pesticides in agriculture, either to control pests or to regulate crop growth has important toxicological implications. After 60 years of use, 2,4-D is still the third most widely used herbicide in the United States and Canada, and the most widely used worldwide both in agricultural and domestic fields to control the growth of broadleaf plants (Industry Task Force II]. 2,4-D mimics the effect of the auxins, or plant growth-regulating hormones, stimulating growth and rejuvenating old cells, and overstimulating young cells, which leads to an abnormal growth pattern and death in some plants (Munro et al., 1992). Exposure to chlorophenoxy herbicides may occur through inhalation, skin contact or ingestion. In most cases, the predominant route of occupational exposure has been by the absorption of spills or aerosol droplets through the skin. Measurements are usually reported in terms of herbicide concentration in the breathing zone air or in the urine of exposed workers. A mean urinary concentration of 1.37 mg/l 2,4-D was measured in workers involved in the production and formulation of 2,4-D herbicide. The highest urinary level of 2,4-D are reported form ground spraying operations in forestry work. According to measurements made in Australia, Canada, Finland, New Zeland, Sweden and USA, mean concentrations ranging form 0.3 to 8 mg/l 2,4-D are common during this type of herbicide application (WHO, 1986).

The extensive use of 2,4-D and the social concerns surrounding it have been catalysts leading to numerous studies seeking evidence of more subtle effects on biochemical mechanisms that might have significance in case of long term or excessive exposure (Dost, 1997). We have previously shown that rats treated with or exposed to 2,4-D exhibit alterations in the myelination rate, ganglioside pattern, dopaminergic and serotoninergic systems and behavior related to these neurotransmitters. Furthermore, 2,4-D exposure decreased litter growth rate from postnatal day 6, when the mothers were treated from gestational day 16 onwards and throughout lactation (Bortolozzi et al., 2002; Duffard et al., 1996; Evangelista de Duffard et al., 1990, 1995; Rosso et al., 2000; Stürtz et al., 2000, 2006).

Along with the altered milk composition, defective maternal behavior may be one of the causes of the reduced growth rate of the litters nursed by 2,4-D treated dams. However, no attempts have been made to study the effect of the herbicide on maternal behavior, in spite of all the evidences mentioned above showing the adverse effects of 2,4 D exposure on neuronal systems and neurotransmitter content.

To address this issue, the present study was designed to investigate the effects of 2,4-D – given to dams at different doses throughout lactation – on serum prolactin, monoamine levels in the arcuate nucleus (AcN), and maternal behavior parameters.

2. Materials and methods

2.1. Animals

Nulliparous female rats, of Wistar origin, obtained from the animal breeding colony of the Pharmacy and Biochemical Faculty, Rosario, Argentina, approximately 3 months of age, were separately placed with fertile males on pro-estrus night, and the presence of spermatozoa was checked in the vaginal smear the following morning. This day was denoted as gestation day 0 (GD 0). After GD 16, pregnant females were individually housed in special zinc breeding cages (40 cm long, 25 cm wide and 10 cm high) in a temperature-controlled nursery (22–24 °C) and maintained on a 12 h light/dark cycle. Food (Purina®, 5002) and water were available *ad lib*.

The cages were divided into two compartments (A and B) by a wire mesh had had a communicating opening that allowed the rats access to both compartments. Each compartment had an individual wire-mesh cover permitting observations of the rat behavior and the introduction of the rats, pups, food and wood shavings. The small compartment (A, 25 cm × 15 cm × 10 cm) was covered by a piece of wood to block the light. The rats usually built their nest in this compartment.

Paper torn into fine strips was provided as nest material on the post partum day 1 (PPD 1). In the behavioral studies, 48 pregnant females were used.

As parturition approached, dams were checked for birth twice daily. The day of delivery was counted as post partum day 0 (PPD 0). All litters delivered by the dams were between 12 and 14 pups. On PPD1, the sex and weight of live newborns were recorded, and each litter was culled to eight pups (four males and four females when possible) in order to standardize litter size and sex ratio of the pups during the postnatal period. Dams were randomly assigned to Treated (T) or Control (C) groups.

Three 2,4-D treated subgroups (12 dams per subgroup with their litter) were included. On PPD1, pellets of food were sprayed with an amount of herbicide calculated to deliver approximately 15, 25 or 50 mg of 2,4-D/kg/day. 2,4-D was dissolved in alcohol solution, mixed with the food and dried before including in the diet (Bortolozzi et al., 1999). The concentration of 2,4-D in food was confirmed by a validated analytical method using gas chromatographic with electron capture detection as described previously (Stürtz et al., 2000). Dietary levels were adjusted based on body weight and food consumption in order to deliver a constant average dietary intake (mg/kg body weight/day). All 2,4-D doses used in this study were less that the non-observed-adverse-effect level (NOAEL) for chronic dietary 2,4-D neurotoxicity in rats established as 77 mg/kg/day (Munro et al., 1992). These doses were selected based on a study where we demonstrated behavioral pattern changes in rats during different periods of development (Bortolozzi et al., 1999), decreases in the content of total lipids, polyunsaturated fatty acid and a change in the content of minor proteins in milk of 2,4-D treated dams (Stürtz et al., 2006).

Food input per animal per cage was maintained at 120 g every 2 days. We did not measure water consumption. Food consumption was recorded as described below:

$$\text{Food consumption} = \text{total food input}(120\,\text{g}) - \text{remaining food at second day}.$$

Control dams were fed with the same diet (6 dams with their litter), sprayed with alcohol and dried, as described for the treated groups but without the herbicide. The amount of food consumption was calculated in the same way as previously described. An extra control group (other 6 dams with their litter) was added where dams were fed *ad libitum* with food without the vehicle.

Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with international laws and approved protocol of the Federation of European Laboratory Animal Science Association (FELASA).

All animals were observed daily for signs of treatment-related effects. During the treatment period, body weights (dam and litter) and food consumption of the dams were recorded every 2 days from PPD 1 to PPD 7. To rule out the possibility of circadian rhythm influence, all animals were sacrificed between 4 and 6 p.m. Dams of each group were killed on PPD 7 by decapitation, after the behavioral test was performed, brains were quickly removed, weighed and stored in tightly capped vials at −76 °C. Trunk blood was collected, allowed to clot at room temperature and the serum was separated and stored at −76 °C until hormonal determinations.

The AcNs were dissected according to Heffner et al. (1980). The tissues were maintained at 0–4 °C throughout the dissection procedure. The tissues were weighed immediately after dissection and stored in sealed containers at −76 °C until further use. Samples were used within 20 days for analysis.

2.2. Reagents

All standards, test chemicals and solvents used in this study were from Sigma Chemical Co. (St. Louis, MO, USA) or Merck (Merck Química Argentina, Bs. As). The 2,4-D acid had a purity of 98%. Chemicals for HPLC analysis were purchased from Baker Chem. (Phillipsburg, USA) and all were of analytical grade.

2.3. Behavioral testing

The choice of maternal behavior parameters observed was made according to Pryce et al. (2001) and Stern (1989). On PPD 5 and PPD 7, the dams were tested for maternal behavior in their home cage. These days were selected since lactation and maternal behavior at these times have been well established. The pups were separated from their mother 15 min before the behavioral tests and maintained at 30 ± 1 °C. Then, the pups were placed in the corner of the large compartment (B) of the cage opposite to the opening leading to the smaller, nesting compartment (A). The maternal behavior was monitored for a period of 10 min between 9:30 and 11:30 a.m. by trained personnel blind to treatment. The following maternal behavior parameters were recorded during the test period:

Nursing behavior with retrieval, crouching, licking (of the whole body of the pups), anogenital licking, time spent out of the nest and eating. We also measured latency and length of retrieval, defined as the time elapsed between the beginning of the experiment and the retrieval of the first and the last pup respectively, and latency of crouching, defined as the time elapsed between the beginning of the experiment and the moment when the mother assumed the crouching position with the pups under her.

2.4. PRL measurement

Serum PRL levels of control and treated dams were measured by double antibody radioimmunoassay using materials generously provided by A. F. Parlow and the NHPP (National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA). PRL was radio-iodinated using the Chloramine T method and purified by passage through Sephadex G75. The results were expressed in terms of the rat prolactin RP-3 standard preparation. Assay sensitivity was 0.5 μg l^{-1} serum and the inter- and intra-assay coefficients of variation were <10% (Jahn et al., 1993).

2.5. Neurochemical analysis

Endogenous monoamines and their metabolites were determined by high-performance liquid chromatography (HPLC) with electrochemical detection (Hallman and Jonsson, 1984). The tissues were placed in Eppendorf tubes, weighed and sonicated in a solution of 0.1 M $HClO_4$ (500 μl). The homogenate was centrifuged at 15,000 rpm for 15 min at 4 °C. A total of 50 μl of supernatant from each sample was injected into a HPLC, and noradrenaline (NA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT) and 5-hydroxyindolacetic acid (5-HIAA) were measured. A standard solution, containing known amine and acid metabolite amounts, was run at the beginning and end of each experiment. The HPLC system was a PM-80 BAS, USA with C-18 reverse-phase column (5 μm particles, 220 mm × 4.6 mm; BAS) and an electrochemical detector with oxidation potential at +0.7 V using a glassy working carbon electrode vs. and Ag/AgCl reference electrode (LC-4C BAS, USA). Mobile phase was composed of 0.15 M citric acid, 0.6 mM sodium octyl sulfate, 4% acetonitrile and 1.6% tetrahydrofuran at pH 3.0; flow rate was fixed at 1.0 ml/min.

3. Statistical analysis

Data are expressed as the means ± S.E.M. Latencies of retrieval, length of retrieval, latencies of crouching and length of activities out of the nest on PND5 and 7 were analyzed using two-way ANOVA followed by Bonferroni post hoc analysis. Analysis of variance (ANOVA), followed by Tukey's post hoc analysis, was used to assess differences due to treatment for mean food consumption, maternal and pups body weight gain, brain monoamine and serum PRL concentrations. To show differences between treatments in the variable number of pups retrieved and number of pups in the nest we used the analysis of χ^2 variable on each test day (PND 5 and PND 7). Percent responding data was analyzed using a Fisher's exact test on each test day (PND 5 and PND 7). Significant differences were established at $P < 0.05$. A Pearson product–moment correlation was used to evaluate possible correlations between brain monoamines levels and PRL level on PPD 7.

4. Results

There were no significant differences between the two control groups (given vehicle-treated or untreated chow) in any of the parameters evaluated, so they were grouped for statistical and description purposes.

There also were no variations either in food or water consumption, between control and treated groups. In accordance with our previous study (Stürtz et al., 2006), mean food consumption (50–90 g every 2 days per dam during test period) and maternal and pup body weight gain were not affected by 2,4-D treatment during the test period, PPD1 to PPD7 (data not shown).

In addition, maternal exposure to 2,4-D had no effect on the number of pups born or on postnatal mortality. The different doses of 2,4-D did not modify the gross general appearance of the dams, including posture and appearance of the coat, nose, eyes and limbs. However, 2,4-D treatment significantly disrupted some aspects of maternal behavior during the test period: PPD 1 to PPD 7 (Table 1).

Although the 2,4-D treatments did not alter nursing behavior, the number of pups retrieved and placed in the nest by each dam on PPD 5, the herbicide significantly increased the mean latency to retrieve the first pup ($p < 0.0001$, $F = 18.45$, degrees of freedom = 1,3) and the length of retrieval of the eight pups ($p < 0.0001$, $F = 31.67$, degrees of freedom = 1,3). Crouching is a maternal behavior associated with nursing; the time spent out of the nest and the latency to assume the crouching position was longer. However, there were no differences between 2,4-D doses (Table 1).

2,4-D reduced the percent of dams that licked their pups in a dose dependent manner and totally annulled the licking of the anogenital region of the pups at all doses (Table 1).

All these alterations in maternal behavior were similar on PPD 7 when was compared to PPD 5. The percentage of the time spent in activities out of the nest was significantly increased in the treated dams with respect to control dams, to levels similar to those observed on PPD 5 (Table 1). Treated dams also showed significant increase in the latency of retrieval and a significant decrease in the number of pups placed in the nest in the treated dams – with a slight dose dependency – was observed (Table 1). Length of retrieval and latencies of crouching did not show dependency with the doses administered, but were significant increased with respect to the control dams. Besides, licking behavior in the treated dams toward their pups was completely blocked at all doses (Table 1).

A remarkable observation was the fact that some of the treated dams ate during the brief test period on both days, while none of the control dams did (Table 1).

Since PRL has been related to the onset and maintenance of the maternal behavior in rats, we measured its serum levels on PPD 7 in control and 2,4-D treated dams. PRL levels on PPD 7 were significantly reduced by 2,4-D treatment at all doses evaluated. This PRL level decrease was 61%, 67% and 71% with respect to control dams, for 15, 25 and 50 mg 2,4-D/kg w groups, respectively (Fig. 1).

Table 1
Maternal behavior parameters on control and 2,4-D treated dams on PPD 5 and PPD 7

	PPD 5	Doses of 2,4-D (mg/kg bw)			PPD 7	Doses of 2,4-D (mg/kg bw)		
	Control	15	25	50	Control	15	25	50
Number of pups retrieved	8.0 ± 0.0	8.0 ± 0.0	6.7 ± 0.3	8.0 ± 0.0	8.0 ± 0.0	8.0 ± 0.0	7.6 ± 0.2	8.0 ± 0.0
Latencies of retrieval	17.3 ± 3.1	37.9 ± 4.4*	40.2 ± 4.4**	38.7 ± 3.7*	26.2 ± 4.8	59.3 ± 6.1***	66.5 ± 7.8***	78.8 ± 7.2***
Length of retrieval of the 8 pups	54.0 ± 2.9	80.8 ± 4.2***	84.7 ± 6.4***	88.0 ± 2.9***	48.9 ± 3.1	87.5 ± 5.7***	95.3 ± 5.2***	93.5 ± 5.6***
Number of pups in the nest	6.4 ± 0.9	5.1 ± 1.0	4.9 ± 1.0	4.2 ± 1.1	8.0 ± 0	2.8 ± 1.1*	1.7 ± 0.9*	1.1 ± 0.7**
Latencies of crouching	216 ± 30	348 ± 32**	328 ± 33*	528 ± 29***	222 ± 26	332 ± 40*	428 ± 37***	600 ± 0***
% of dams licking pups	100.0	50.0***	42.0***	16.0***	100.0	0.0***	0.0***	0.0***
% of dams licking ano-genital region of the pups	83.0	0.0***	0.0***	0.0***	75.0	0.0***	0.0***	0.0***
% of dams leaving the nest	25.0	100.0***	100.0***	100.0***	42.0	67.0*	83.0***	100.0***
Time spent out of the nest	13 ± 8	132 ± 10***	148 ± 8***	138 ± 11***	26 ± 12	136 ± 23***	141 ± 24***	120 ± 10***
% of dams going out to eat	0.0	17.0	25.0	33.0	0.0	17.0	25.0	17.0

There were no statistical differences between the control values on PPD 5 and PPD 7. Time is expressed in seconds. Significant differences from respective controls: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

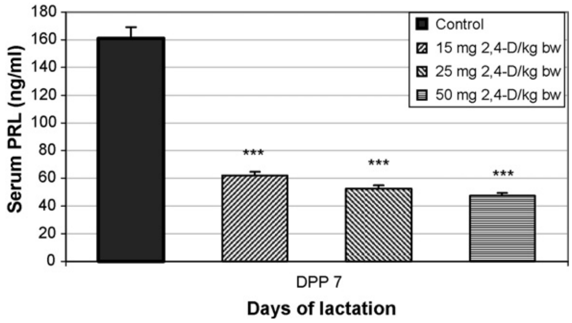

Fig. 1. Serum Prolactin levels on PPD 7 in control and 2,4-D treated dams. Significantly different from control: ***$P < 0.001$.

AcN neurotransmitter levels on PPD 7 were also altered by 2,4-D. 5-HT and 5-HIAA levels were diminished by all doses of 2,4-D in a dose dependent manner (Table 2). Treatment with 50 or 25 mg 2,4-D/kg bw increased DA, DOPAC and HVA levels in the AcN of the dams, without significantly modifying NA content (Table 2). However, the lower dose of the herbicide had no significant effects on DA, NA or their metabolites.

When the DA and 5-HT levels for the different treatment groups are plotted as a function of the PRL level for each group on PPD 7 for individual test animals, a significant positive correlation was found (r^2 0.9975 for DA and r^2 0.9970 for 5-HT; P = for both DA and 5-HT = 0.015).

5. Discussion

The aim of this study was to examine whether disruptions in maternal behavior produced by 2,4-D exposure play a role in the 2,4-D-associated changes in exposed neonate rats previously reported by us, particularly in the impaired growth rate of the neonates (Bortolozzi et al., 1998; Stürtz et al., 2006).

Maternal care is a complex, spontaneous, instinctive, and species-specific behavior determined by physiological modifications that occur just prior to and immediately after delivery and it is crucial for the survival of mammalian pups (Stern, 1989). Different authors have reported that decreased or absent contact between mothers and pups can influence the behavioral development of the latter, especially in coping with stress and disease (Caldji et al., 1998; Lovic et al., 2001).

The present findings indicate that 2,4-D impaired maternal behavior. Thus, pup care parameters, such as licking and grouping of the pups were decreased and the latencies of retrieval and crouching were increased, as well as the time spent by the mothers outside the nesting area. However, pups did not show body weight loss during the test period; probably because milk supply was normal.

Pup retrieval behavior is regulated by multisensory processes and can be considered as a chain of motor responses elicited by a variety of stimuli emanating from the female and/or pups which promotes orientation, attention and arousal (Stern, 1990). The initial stimuli induce the approximation of the mother to the pups and perioral trigeminal stimulation elicits the retrieval and grouping of the pups in the nest (Stern and Kolunie, 1989, 1991). Whole body licking and ano-genital licking are also complex motor behaviors regarded as active pronurturant behaviors, triggering the behavioral sequence leading to lactation (Stern and Lonstein, 2001) in the mother and inducing micturition and defecation in the pups.

Since maternal behavior does not depend exclusively on one neurotransmitter or hormonal system, but results from the complex interaction between mother and pups, we hypothesized that 2,4-D may decrease the motivation to care for the pups, and thereby increase the dams‘activities out of the nest. 2,4-D may produce this through interference with the sensory processes implicated in the perception of the pups, which in turn may decrease motivation. Alternatively, 2,4-D may affect not only the mother but also the pups which could show deficient signaling toward their mother.

On the other hand, serum PRL content is constantly elevated in lactating females. It has been demonstrated that PRL is involved in the expression of all the components of maternal behavior, acting in the medial preoptic nucleus (Bridges, 1994; Bridges et al., 1990, 1997). The decrease in serum PRL observed in the treated dams (Fig. 1) may be other causal factor involved in the disruption of maternal behavior.

In addition, lactation is characterized by an inhibition of the hypothalamic-tuberoinfundibular dopaminergic (TIDA) pathways of the AcN and an increase in serotoninergic function, both factors that contribute to the hyperprolactinemia induced by suckling (Ben-Jonathan et al., 1989; Boyd and Reichlin, 1978). The suckling stimulus results in an acute diminution of the amount of dopamine released into the portal blood that arrives to the anterior pituitary gland (Freeman et al., 2000), thereby inducing acute PRL release, since DA is the main PRL inhibitory factor. Furthermore, during late pregnancy and lacta-

Table 2
AcN Catecholamines and Indoleamines levels in control and 2,4-D treated dams on PPD 7

	Control	Doses of 2,4-D (mg/kg bw)		
		15	25	50
NA	72.0 ± 6.5	49.9 ± 4.5	50.6 ± 4.5	51.1 ± 8.4
DA	35.5 ± 1.5	41.3 ± 2.0	49.9 ± 1.5*** (↑ 40%)	51.7 ± 1.9*** (↑ 46%)
DOPAC	10.4 ± 0.7	9.4 ± 2.1	17.2 ± 1.8* (↑ 65%)	18.6 ± 0.9** (↑ 79%)
HVA	14.5 ± 1.7	25.8 ± 3.2*(↑ 78%)	25.5 ± 2.1* (↑ 76%)	28.1 ± 2.4* (↑ 94%)
5-HT	252.1 ± 3.0	71.1 ± 1.9*** (↓ 72%)	40.4 ± 1.7*** (↓ 84%)	30.9 ± 0.9*** (↓ 88%)
5-HIAA	85.8 ± 3.5	59.7 ± 2.5*** (↓ 30%)	40.6 ± 1.5 *** (↓ 53%)	38.7 ± 2.7*** (↓ 55%)

Catecholamine and Indoleamine levels are expressed in fmol/mg wet weight. The percentage of increase with respect to the control group is shown between brackets. Significant differences from controls: *$p < 0{,}05$, **$p < 0{,}01$ and ***$p < 0{,}001$.

tion there is a decrease in TIDA neuron dopaminergic activity and the capacity of elevated PRL to stimulate DA synthesis and release to the portal vessels (short loop feedback) is impaired (Andrews et al., 2001; Andrews, 2005; Arbogast and Voogt, 1996). This disruption of the normal PRL negative feedback appears to be fundamental for the development of the lactational hyperprolactinemia necessary for optimal milk synthesis (Andrews, 2005). The increased levels of DA, DOPAC and HVA in the AcN in the 2,4-D treated dams may indicate a persistence of high TIDA neuron activity during lactation and hence, a diminished suckling-induced PRL release. On the other hand, the impaired maternal behavior observed in the treated dams may result in a decreased suckling stimulus and consequently, higher TIDA neuron dopaminergic tone, since the suckling stimulus suppresses TIDA neuronal activity (Freeman et al., 2000; Li et al., 1999).

It has been demonstrated that the serotoninergic neurons of the dorsal raphe that project to hypothalamic region participate in the neuroendocrine pathways that mediate the suckling induced PRL release (Barofsky et al., 1983; Di Renzo et al., 1989; Jahn et al., 1999). The drastic diminution of 5-HT and 5-HIAA observed in the 2,4 D treated dams may also be responsible for the diminished circulating PRL, along with the increased DA levels. Additionally, this disruption in neurotransmitter balance may play a role in the alteration of maternal behavior parameters.

In conclusion, 2,4-D produced disruption of some maternal behavior parameters that were associated with decreased 5-HT and increased DA levels in the AcN and decreased circulating PRL. The altered 5-HT and DA in the AcN may be responsible for the diminution of serum PRL and all three play a role in the altered maternal behavior. In turn, the disrupted maternal care and lactation may contribute, along with the direct exposure to the herbicide through the mother's milk, to posterior developmental, neural and behavioral defects observed in the offspring. These "cross-generational" effects must be taken into account when evaluating toxicological effects of other extensively used xenobiotics that usually are considered as not producing developmental effects. In addition, we want to highlight that a contribution of this study is to document effects of 2,4-D on the care received by the developing organism, and to call attention to the potential pivotal role that the alteration of maternal behavior may have in the developmental outcomes of toxicology studies.

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References

Andrews, Z.B., Kokay, I.C., Grattan, D.R., 2001. Dissociation of prolactin secretion from tuberoinfundibular dopamine activity in late pregnant rats. Endocrinology 142, 2719–2724.

Andrews, Z.B., 2005. Neuroendocrine regulation of prolactin secretion during late pregnancy: easing the transition into lactation. J. Neuroendocrinol. 17, 466–473.

Arbogast, L.A., Voogt, J.L., 1996. The responsiveness of tuberoinfundibular dopaminergic neurons to prolactin feedback is diminished between early lactation and midlactation in the rat. Endocrinology 137, 47–54.

Barofsky, A.L., Taylor, J., Massari, V.J., 1983. Dorsal raphe-hypothalamic projections provide the stimulatory serotonergic input to suckling-induced prolactin release. Endocrinology 113, 1894–1903.

Ben-Jonathan, N., Arbogast, L.A., Hyde, J.F., 1989. Neuroendocrine regulation of prolactin release. Prog. Neurobiol. 33, 399–447.

Bortolozzi, A., Duffard, R.O., Rubio, M., Stürtz, N., Evangelista de Duffard, A.M., 1998. Regionally specific changes in central brain monoamine levels by 2,4-dichlorophenoxyacetic acid in acute treated rats. Neurotoxicology 19, 839–851.

Bortolozzi, A., Duffard, R., Evangelista de Duffard, A.M., 1999. Behavioral alterations induced in rats by a pre- and postnatal exposure to 2,4-dichlorophenoxyacetic acid. Neurotoxicol. Teratol. 21, 451–465.

Bortolozzi, A., Duffard, R., Antonelli, M., Evangelista de Duffard, A.M., 2002. Increased sensitivity in dopamine D(2)-like brain receptors from 2,4-dichlorophenoxyacetic acid (2,4-D)-exposed and amphetamine-challenged rats. Ann. N.Y. Acad. Sci. 965, 314–323.

Boyd, A.E., Reichlin, S., 1978. Neural control of prolactin secretion in man. Psychoneuroendoc. 3, 113–130.

Bridges, R.S., 1994. The role of lactogenic hormones in maternal behavior in female rats. Acta Paediatr. Suppl. 397, 33–39.

Bridges, R.S., DiBiase, R., Loundes, D.D., Doherty, P.C., 1985. Prolactin stimulation of maternal behavior in female rats. Science 227, 782–784.

Bridges, R.S., Numan, M., Ronsheim, P.M., Mann, P.E., Lupini, C.E., 1990. Central prolactin infusions stimulate maternal behavior in steroid-treated, nulliparous female rats. Proc. Natl. Acad. Sci. 87, 8003–8007.

Bridges, R.S., Robertson, M.C., Shiu, R.P., Friesen, H.G., Stuer, A.M., Mann, P.E., 1996. Endocrine communication between conceptus and mother: placental lactogen stimulation of maternal behavior. Neuroendocrinology 64, 57–64.

Bridges, R.S., Robertson, M.C., Shiu, R.P., Sturgis, J.D., Henriquez, B.M., Mann, P.E., 1997. Central lactogenic regulation of maternal behavior in rats: steroid dependence, hormona specificity, and behavioral potencies of rat prolactin and rat placental lactogen I. Endocrinology 138, 756–763.

Caldji, C., Tannenbaum, B., Sharma, S., Francis, D., Plotsky, P.M., Meaney, M.J., 1998. Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. Proc. Natl. Acad. Sci. 95, 5335–5340.

Crews, D., Willingham, E., Skipper, J.K., 2000. Endocrine disruptors: present issues, future directions. Q. Rev. Biol. 75, 243–260.

Di Renzo, G., Amoroso, S., Taglialatela, M., Canzoniero, L., Basile, V., Fatatis, A., Annunziato, L., 1989. Pharmacological characterization of serotonin receptors involved in the control of prolactin secretion. Eur. J. Pharmacol. 162, 371–373.

Dost, F.N., 1997. Toxicology and potential health risk of chemicals that may be encountered by workers using forest vegetation management options. Part III, Risk to workers using 2,4-D formulations. National Library of Canada Cataloguing in Publication Data "Title number 5".

Duffard, R., García, G., Rosso, S., Bortolozzi, A., Madariaga, M., DiPaolo, O., Evangelista de Duffard, A.M., 1996. Central nervous system myelin deficit in rats exposed to 2,4-dichlorophenoxyacetic acid throughout lactation. Neurotoxicol. Teratol. 18, 691–696.

Evangelista de Duffard, A.M., Bortolozzi, A., Duffard, R.O., 1995. Altered behavioral responses in 2,4-dichlorophenoxyacetic acid treated and amphetamine challenged rats. Neurotoxicology 16, 479–488.

Evangelista de Duffard, A.M., Orta, C., Duffard, R., 1990. Behavioral changes in rats fed a diet containing 2,4-dichlorophenoxyacetic butyl ester. Neurotoxicology 11, 563–572.

Freeman, M.E., Kanyicska, B., Lerant, A., Nagy, G., 2000. Prolactin: structure, function, and regulation of secretion. Physiol. Rev. 80, 1523–1631.

González-Mariscal, G., Chirino, R., Flores-Alonso, J.C., Rosenblatt, J.S., Beyer, C., 2004. Intracerebroventricular injections of prolactin counteract the antagonistic effect of bromocriptine on rabbit maternal behaviour. J Neuroendocrinol. 16, 949–955.

Hallman, H., Jonsson, G., 1984. Neurochemical studies on central dopamine neurons. Regional characterization of dopamine turnover. Med. Biol. 62, 198–209.

Hansen, S., 1994. Maternal behavior of female rats with 6-OHDA lesions in the ventral striatum: characterization of the pup retrieval deficit. Physiol. Behav. 55, 615–620.

Hansen, S., Harthon, C., Wallin, E., Lofberg, L., Svensson, K., 1991. The effects of 6-OHDA-induced dopamine depletions in the ventral or dorsal striatum on maternal and sexual behavior in the female rat. Pharmacol. Biochem. Behav. 39, 71–77.

Heffner, T.G., Hartman, J.A., Seiden, L.S., 1980. A rapid method for the regional dissection of the rat brain. Pharmacol. Biochem. Behav. 13, 453–456.

Industry Task Force II on 2,4-D research date web site (1999). http://www.24d.org.

Jahn, G.A., Rastrilla, A.M., Deis, R.P., 1993. Correlation of growth hormone secretion during pregnancy with circulating prolactin in rats. J. Reprod. Fertil. 98, 327–333.

Jahn, G.A., Kalia, V., Hole, D., Wilson, C.A., Deis, R.P., 1999. Receptors and neurotransmitters involved in the dual modulation of prolactin release by the serotoninergic system in pregnant and lactating rats. J. Reprod. Fertil. 116, 261–268.

Johns, J.M., Noonan, L.R., Zimmerman, L.I., Li, L., Pedersen, C.A., 1994. Effects of chronic and acute cocaine treatment on the onset of maternal behavior and aggression in Sprague–Dawley rats. Behav. Neurosci. 108, 107–112.

Johns, J.M., Noonan, L.R., Zimmerman, L.I., Li, L., Pedersen, C.A., 1997. Effects of short and long term withdrawal from gestational cocaine treatment on maternal behavior and aggression in Sprague–Dawley rats. Dev. Neurosci. 19, 368–374.

Keer, S.E., Stern, J.M., 1999. Dopamine receptor blockade in the nucleus accumbens inhibits maternal retrieval and licking, but enhances nursing behavior in lactating rats. Physiol. Behav. 67, 659–669.

Li, C., Chen, P., Smith, M.S., 1999. Neuropeptide Y and tuberoinfundibular dopamine activities are altered during lactation: role of prolactin. Endocrinology 140, 118–123.

Lovic, V., Gonzalez, A., Fleming, A.S., 2001. Maternally separated rats show deficits in maternal care in adulthood. Dev. Pshychobiol. 39, 19–33.

Miller, S.M., Lonstein, J.S., 2005. Dopamine d1 and d2 receptor antagonism in the preoptic area produces different effects on maternal behavior in lactating rats. Behav. Neurosci. 119, 1072–1083.

Munro, I.C., Carlo, G.L., Orr, J.C., Sund, K., Wilson, R.M., Kennepohl, E., Lynch, B., Jablinske, M., Lee, N., 1992. A comprehensive, integrated review and evaluation of the scientific evidence relating to the safety of the herbicide 2,4-D. J. Am. Coll. Toxicol. 11, 559–664.

Numan, M., 1994. Maternal behavior. In: Knobil, E., Neill, J.D. (Eds.), The Physiology of Reproduction, 2nd ed. Raven Press, New York, pp. 221–302.

Palanza, P., Howdeshell, K.L., Parmigiani, S., vom Saal, F.S., 2002a. Exposure to a low dose of bisphenol A during fetal life or in adulthood alters maternal behavior in mice. Environ. Health Perspect. 110, 415–422.

Palanza, P., Morellini, F., Parmigiani, S., vom Saal, F.S., 2002b. Ethological methods to study the effects of maternal exposure to estrogenic endocrine disrupters: a study with methoxychlor. Neurotoxicol. Teratol. 24, 55–69.

Pryce, C.R., Bettschen, D., Feldon, J., 2001. Comparison of the effects of early handling and early deprivation on maternal care in the rat. Dev. Psychobiol. 38, 239–251.

Rosenblatt, J.S., Mayer, A.D., Siegel, H.I., 1985. Maternal behavior among the nonprimate mammals. In: Adler, N., Plaff, D., Goy, R.W. (Eds.), Handbook of Behavioral Neurobiology, vol. 7. Plenum Press, New York, pp. 229–298.

Rosso, S.B., Cáceres, A.O., de Duffard, A.M., Duffard, R.O., Quiroga, S., 2000. 2,4-Dichlorophenoxyacetic acid disrupts the cytoskeleton and disorganizes the Golgi apparatus of cultured neurons. Toxicol. Sci. 56, 133–140.

Simmons, S.L., Cummings, J.A., Clemens, L.G., Núñez, A.A., 2005. Exposure to PCB 77 affects the maternal behavior of rats. Physiol. Behav. 84, 81–86.

Stern, J.M., 1989. Maternal behavior. In: Brush, L.S.E.R. (Ed.), Psychoendocrinology. Academic Press, San Diego, pp. 105–226.

Stern, J.M., 1990. Multisensory regulation of maternal behavior and masculine sexual behavior: a revised view. Neurosci. Biobehav. Rev. 14, 183–200.

Stern, J.M., Kolunie, J.M., 1989. Perioral anesthesia disrupts maternal behavior during early lactation in Long–Evans rats. Behav. Neural Biol. 52, 20–38.

Stern, J.M., Kolunie, J.M., 1991. Trigeminal lesions and maternal behavior in Norway rats: I. Effects of cutaneous rostral snout denervation on maintenance of nurturance and maternal aggression. Behav. Neurosc. 105, 984–997.

Stern, J.M., Lonstein, J.S., 2001. Neural mediation of nursing and related maternal behaviors. Prog. Brain Res. 133, 263–278.

Stürtz, N., Evangelista de Duffard, A.M., Duffard, R., 2000. Detection of 2,4-dichlorophenoxyacetic acid (2,4-D) residues in neonates breast-fed by 2,4-D exposed dams. Neurotoxicology 21, 147–154.

Stürtz, N., Bongiovanni, B., Rassetto, M., Ferri, A., Evangelista de Duffard, A.M., Duffard, R., 2006. Detection of 2,4-dichlorophenoxyacetic acid in rat milk of dams exposed during lactation and milk analysis of their major components. Food Chem. Toxicol. 44, 8–16.

Suomi, S.J., 1997. Early determinants of behaviour: evidence from primate studies. Br. Med. Bull. 53, 170–184.

Taylor, M.R., Harrison, P.T.C., 1999. Ecological effects of endocrine disruption: current evidence and research priorities. Chemosphere 39, 1237–1248.

WHO 1986, IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol 41.