Effect of 2,4-dichlorophenoxyacetic acid on milk transfer to the litter and prolactin release in lactating rats

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ABSTRACT
The effects of 2,4-dichlorophenoxyacetic acid (2,4-D) on brain monoamines and the serum level of hormones involved in milk synthesis and on the milk ejection reflex in rats were evaluated. Dams were treated with 2.5, 5, 15, 25, 50 or 70 mg 2,4-D/kg bw according to two experimental designs: (a) through food from post partum day 1 (PPD 1) to PPD 16 and the respective control groups or (b) an unique i.p. injection on PPD 11. To measure milk ejection, the litter was separated from the mother at the 11th day of lactation during 8 h, returned to their mothers and allowed to suckle for a period of 15 min. The procedure was repeated on 3 consecutive days until the end of treatment. The change in litter weight during the suckling period was taken as a measure of the amount of milk ejected during this period. The dams’ serum prolactin (PRL), oxytocin (OT) and growth hormone levels were determined by radioimmunoassay. Both treatment regimens produced a dose-dependent decrease in the amount of milk ejected and circulating PRL and OT secreted in response to the suckling stimulus. Administration of OT before returning the pups restored the milk ejection, indicating no impairment in the capacity of the mammary gland to produce and secrete milk. In addition, dopamine levels were increased by the 2,4-D treatments in arcuate nucleus (ArN) and anterior lobe of pituitary gland (AL), while serotonin level was drastically decreased in ArN. 2,4-D treatment increased both calcium-dependent and calcium-independent nitric oxide synthase (NOS) activities in ArN. These results suggest that 2,4-D inhibits the suckling-induced hormone release, milk transfer to the litter at the central level, through a stimulation of hypothalamic NOS and dopamine and by an inhibition of hypothalamic serotonin transmission.

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1. Introduction

A large amount of data indicates that substances naturally present in the environment or of anthropogenic origin (the endocrine disruptors (EDs)) are able to interfere with the functioning of the endocrine system in vertebrates (Crews et al., 2000; Taylor and Harrison, 1999). They may mimic, block, or modulate the synthesis, release, transport, metabolism, binding, or elimination of natural hormones (Gore, 2001). The results of endocrine disruption are often not easily detected. EDs have been shown to disrupt embryonic development, sexual differentiation, reproduction, immune function, behaviour, other responses mediated by hormones (Panzica et al., 2005).

Lactation is the final step of the reproductive cycle. The endocrine control of lactation is a complex physiological process that involves a wide array of hormones, growth factors, neurotransmitters and proteins for the proper regulation of mammogenesis, lactogenesis, galactopoiesis and galactokinesis (Buhimschi, 2004). This provides multiple targets for the action of xenobiotics (EPA/630/R-96/012). Once lactation has been established, it is maintained by the suckling stimulus, that produces the removal of milk from the mammary gland and the secretion of the hormones involved in milk ejection, namely oxytocin, and in milk secretion, mainly prolactin (PRL). Suckling produces a neural signal sent from the nipples to the hypothalamus, where it induces release of oxytocin (OT) and PRL. OT acts by inducing contraction of the myoepithelial cells and voiding of milk, while PRL stimulates milk synthesis (Tucker, 2000). Any modifications produced on these systems could have consequences on the amount and quality of milk produced by the dams. This fact is crucial during lactation because in most mammals, milk is the only source of energy for the pups during this period. Although milk transfer to the litter may not be considered a classical indicator of toxicity, if any environmental contaminant has the capacity of negatively affecting milk transfer
to the pups of the mammalian species, man included, that come in contact with it, this certainly can result in detrimental effects on the health and survival of the species. This sole fact justifies the study of the impact of environmental contaminants on lactational performance, independently of whether this effect is due to adverse pharmacological or to toxicological responses.

2.4-Dichlorophenoxyacetic acid is a systemic chlorophenoxy herbicide widely used during the last 60 years to control broadleaf weeds in cereal cropland and on industrial property, lawns, turf, pastures and gardens (Munro et al., 1992). Sales of this herbicide have remained fairly constant in the US and Western Europe over the last decade, but have increased in a number of developing countries, particularly Argentina and Brazil. Exposure to this chemical through agricultural use, food products, or through lawn and garden use has been demonstrated in several studies (Kohli et al., 1974; Taskar et al., 1982; Harris et al., 1992).

Little is known about possible direct effects of 2,4-D during the lactation period, specially on the production and ejection of milk. In previous studies we have demonstrated that 2,4-D is transferred to the neonates through mothers’ milk and causes a dose-dependent decrease on the pup’s body weight gain, a reduction on milk content of fatty acids with a chain length of 20 carbons or more and an alteration in the pattern of milk proteins, suggesting that 2,4-D may have some deleterious effect on mammary gland function (Stürtz et al., 2000, 2006).

In mammals, PRL release is mainly under inhibitory control exerted by dopamine (DA) originating in the neurons of the arcuate and periventricular nuclei, but a great variety of hypothalamic and non-hypothalamic factors also participate in this regulation. It has been demonstrated that the sucking stimulus results in a decrease of DA released into portal blood (de Groot et al., 1981) that arrives at the anterior pituitary gland (Freeman et al., 2000). In addition, it has also been shown that serotoninergic pathways originating in the raphe nuclei mediate the sucking-induced PRL release (Fessler et al., 1984; Van de Kar and Bethea, 1982; Jahn and Deis, 1994), 5-HT mechanisms of action may involve stimulation of some PRL-releasing factors (Clemens et al., 1978; Garthwaite and Hagen, 1979) or inhibition of the PRL-inhibiting hormone secreted from the hypothalamus (Lynch et al., 1984; Pliotte and Porter, 1981).

On the other hand, nitric oxide (NO) has been shown to play a regulatory role in neuroendocrine function and PRL secretion (Bram et al., 1997). NO inhibits PRL secretion, both in vivo and in vitro (Duvalinsky et al., 1995; Lafuente et al., 2004) at anterior pituitary level. It is synthesised from l-arginine by nitric oxide synthase (NOS) (Dawson and Snyder, 1994; Knowles et al., 1989). Within the hypothalamus, NOS activity is found in the paraventricular and supraoptic nuclei as well as in the lamina terminalis (Bhat et al., 1995; Brede et al., 1991). Moderate activity is found in the medial preoptic nucleus, ventromedial nucleus, suprachiasmatic nucleus, and in median eminence (Bhat et al., 1995).

The goal of the present study was to determine whether 2,4-D alters milk transfer to the litter, and if so, if this alteration could be linked to changes in the serum hormone and brain monoamine levels and/or NOS activities related to these processes.

2. Materials and methods
2.1. Animals and exposure to 2,4-D

Nulliparous female rats of Wistar origin, between 90 and 110 days old and weighing approximately 180–210 g were obtained from the animal breeding colony of the Pharmacy and Biochemical Faculty, Rosario, Argentina. Stages of the reproductive cycle were monitored via daily cytological examination of vaginal smears. They were mated individually with fertile males on the night of pro-oestrus. Mating was confirmed by observing sperm in smears on the morning following mating. This day was designated as gestational day 0 (GD 0). On GD 16, females were housed individually in plastic breeding cages in a temperature-controlled nursery (22 ± 2 °C) and maintained on a 12 h light–dark cycle (8 am–8 pm light). Rat lab chow (Purina® 5002) and water were available ad libitum. 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 PPD 1, 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. All experimental procedures were performed in accordance with the guidelines for the use and care of laboratory animals as framed by the approved protocol of the Federation of European Laboratory Animal Science Association (FELASA).

All standards, test chemicals and solvents used were obtained from Sigma Chemical Co. (St. Louis, MO, USA) or Merck (Merck Quimica Argentina). The 2,4-D acid had a purity of 98%.

2.2. Experimental designs

2.2.1. Experiment 1

Dams were treated with a daily oral dose in the diet of about 2.5, 5, 10, 15, 25, 50 or 70 mg/kg of 2,4-D from PPD 1 until PPD 16. It was dissolved in alcohol, mixed with the food and dried before giving the food to the dams according to Bortolozzi et al. (1999, 2003). 2,4-D concentration in food was confirmed by a validated analytical method using gas chromatographic separation with electron capture detection. Dietary levels were adjusted based on the most recent dam body weight and food consumption in order to deliver a constant average dietary intake (mg 2,4-D/kg weight per day) (Stürtz et al., 2000).

Control dams were fed with the same diet (six dams with their litter), sprayed with vehicle 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 (Stürtz et al., 2006). An extra control group (six dams with their litter) was added in which dams were fed at libitum with food without the vehicle.

All animals were observed daily for signs of treatment-related effects. During the treatment period, body weights (dams and litters) and food consumption of the dams were recorded every 2 days from PPD 1 to PPD 16.

In order to determine the response to suckling, on days 11–13 of lactation, control or 2,4-D-treated mothers were isolated from the litter at 08.00 h. The litter was weighed to the nearest 0.1 g and returned to their mothers at 17:00 h. This procedure was repeated on 3 consecutive days and only the results obtained on the third day were recorded. This method was used to habituate the rats to the procedure and thus minimise the stress produced by the isolation of the mother from the litter, which can interfere with PRL and OT release (Taleisnik and Deis, 1964). On PPD 12, before returning the pups to their mothers, blood samples were obtained from the tail vein of the dams under light ether anaesthesia, in order to determine basal serum concentrations of growth hormone (GH), PRL and OT. On PPD 13, after 15 min of suckling, the mothers were bled from the tail vein under light anaesthesia and the litters weighed again. The gain in weight of the litter during the time of sucking on PPD 13 was taken as measure of the amount of milk ejected by the dams. Serum was separated and stored at −30 °C. PRL, GH and OT levels were determined by RIA.

On PPD 16, all dams were killed by decapitation, between 17:00 and 18:00 h, to rule out the possibility of influence of circadian rhythms. Brains were rapidly removed and/or NOS activities related to these processes.

2.2.2. Experiment 2

Forty-eight lactating dams weighing 210–240 g at the beginning of lactation with their eight suckling pups were used to perform this experiment; rat chow and tap water were freely available. At 18:00 h on PPD 11, they were divided into six experimental subgroups and received an intra perioneal injection of either vehicle or 2.5, 5, or 15 or 50 mg 2,4-D/kg bw and subjected to the acute suckling experiment as described on Experiment 1. On PPD 16 dams were killed by decapit-ation and brain areas were removed and processed as described above for Experiment 1.

2.2.3. Experiment 3

To determine if administration of OT before returning the pups to the treated dams can restore normal milk ejection, 24 lactating dams weighing 210–240 g at the beginning of lactation, suckling eight pups and subjected to the acute suckling procedure as described above, were divided into three experimental groups. On PPD 11, they received an intra perioneal injection of saline solution (n=8) or 50 mg 2,4-D/kg bw (n=16). On PPD 13 5 min before the start of the suckling period the treated dams were injected with 100 mU OT (Syntocinon, Novartis, Argentina) in 0.3 ml saline (n=8) or saline only (n=8). The rest of the procedure was performed as described on Experiment 1.
2.3. Hormone determinations

PRL and GH were measured by double antibody radioimmunoassay using materials generously provided by Dr. Parlow and the NHPP (National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA, USA). The hormones were radio-ioted using the Chloramine T method and purified by passage through Sephadex G75. The results were expressed in terms of the rat PRL RP-3 or rat GH RP-2 standard preparations. Assay sensitivity was 0.5 µg L\(^{-1}\) serum and the inter- and intra-assay coefficients of variation were less than 10% for all hormones. OT was measured by double antibody radioimmunoassay using an antibody and purified OT generously provided by Dr. N. Hagino and Novartis Argentina respectively. The hormone was radio-ioted using the Chloramine T method and purified by passage through Sephadex G50. The standard curve was prepared using the same preparation of purified OT used for radioiodination. To maximize sensitivity of the assay, the standards and serum samples were incubated 24 h with appropriate dilution of the antibody, subsequently the labeled hormone (8–10\(^{12}\) cpms) was added and the tubes incubated overnight before addition of second antibody. Assay sensitivity was 8 ng L\(^{-1}\) serum and the intra-assay coefficients of variation were less than 10%. All the samples were measured on the same assay by duplicate.

2.4. Neurochemical analysis

Levels of endogenous monoamines and their metabolites were determined on hypothalamic accrete nucleus (AN) and anterior lobe (AL) by high-performance liquid chromatography (HPLC) with electrochemical detection (Hallman and Jonsson, 1984). Tissues were placed in Eppendorf tubes, weighed and sonicated in a solution of 0.1 M HClO4 (500 µl). The homogenate was centrifuged at 15,000 rpm for 15 min at 4 °C. Noradrenaline (NA), dopamine (DA), 3,4-dihydroxyphenyletylic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT) and 5-hydroxyindolacetic acid (5-HIAA) were measured in 50 µl of the supernatants by HPLC (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 glass working carbon electrode vs. and Ag/AgCl reference electrode (LC-4C BAS, USA). A standard solution, containing known amine and acid metabolite amounts, was run at the beginning and end of each experiment. 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.

2.5. Assay of nitric oxide synthase activity

NOS activity was assayed by measuring the conversion of L-[U-14C]Citrulline according to the method of Bredt and Snyder (1990) with some modifications (Weiner et al., 1994). Briefly, frozen AN were homogenized in 1.5 ml ice-cold buffer containing 20 mM Heps, pH 7.4; 1 mM EDTA; 1 mM dithiothreitol (DTT); 1 mM NADPH. Protein concentration was determined by the Bradford method (1976) on a 20 µl aliquot of the crude homogenate. Homogenates were centrifuged at 12,000 × g for 15 min at 4 °C. NOS activity was determined in duplicate within 1 h of preparation. At 300 µl supernatant were added to 200 µl reaction buffer, containing final concentrations of 50 mM Heps, pH 7.4; L-[U-14C]Citruline (250 000 cpm, specific activity 330 mCi/mmol, NEN, Boston, MA, USA); 1 mM NADPH; 1.25 mM CaCl\(_2\); 1 mM EDTA; 1 mM DTT; and 10 µM tetrahydrobiopterin. The reaction mixture was incubated for 15 min at 37 °C and the reaction terminated by adding 1.5 ml stop buffer (20 mM Heps, pH 5.5; 2 mM EDTA). Samples were applied to 1 ml Dowex AG50W-X8 (Na\(^{+}\) form) columns pre-equilibrated with stop buffer, L-[U-14C]Citruline was eluted with 2.5 ml water and concentrated in a Beckman (Fullerton, CA, USA) scintillation counter (Model LS1701). Calcium-dependent activity was calculated by subtracting from total activity of each sample (measured with calcium) the samples measured with EGTA (without calcium) that were considered calcium-independent activity. The assay sensitivity was 0.05 pmol NO/min/mg protein.

2.6. Statistical analysis

All data are presented as mean ± SEM. Statistically differences between groups were assessed using analysis of variance (ANOVA) followed by the Tukey test for multiple comparisons test. Where significant differences were found, means were tested using the Student–Newman–Keuls test for multiple comparisons; a value of p ≤ 0.05 was considered statistically significant. The body weight gain differences between groups were analyzed using two-way ANOVA, followed by Turkey's post hoc analysis. A Parson product-moment correlation was used to evaluate possible correlations between brain monoamines levels and hormone levels on PPD 16. Simple regression analysis was performed to evaluate possible relationship between the amount milk ejected and the different doses of the herbicide.

3. Results

3.1. Clinical examination

Acute or chronic administration of 2,4-D at any dose did not affect adversely the general condition and body weight gain of the
dams and any pups died during the test period. The treatments did not affect mean food consumption either. As no significant differences were observed between both control groups of dams, we grouped them together.

3.2. Pups body weights

The chronically treated groups (Experiment 1) had a significant reduction ($p < 0.001$) in body weight, with the greatest effects observed in the 50 and 70 mg 2,4-D/kg bw treated groups. This effect was observed after PPD 7 in all treated groups except the 2.5 mg 2,4-D/kg bw group, in which the effect became significant on day 10. After the acute, i.p. injection, administration of the herbicide, only the pups of dams treated with 25 or 50 mg 2,4-D/kg bw showed a significant decrease in the body weight gain (Table 1).

3.3. Effect of acute or chronic 2,4-D treatment on the response to the suckling stimulus

The milk transfer, determined by gain in pup weight is shown in Fig. 1. 2,4-D decreased in a dose-related manner the quantity of milk obtained by the pups from dams given the chronic (Experiment 1) or acute (Experiment 2) treatments ($p < 0.001$) compared with the controls (Fig. 1A and B). Evaluation of the dose response curve by linear regression showed a strong correlation for both treatments ($r^2 = 0.98$, $p < 0.0001$; $r^2 = 0.95$, $p < 0.005$; for Experiment 1 and Experiment 2, respectively). The acute treatment (Experiment 2) was more effective in blocking the suckling response than the chronic administration of the herbicide (Experiment 1). The decreased response observed in the 2,4-D treated dams could not be the consequence of less suckling by their pups, as they attached themselves readily to the nipples and sucked vigorously during the 15 min test period. Most of the pups were still attached to the nipples when the mothers were removed for collection of blood samples. All the nipples showed signs of recent and intense suckling and the pups from 2,4-D treated mothers, although smaller than control pups, were very active. In these respects, there were no differences between the control litters and litters from 2,4-D treated mothers or in the behaviour of the mothers during the 15 min suckling period. Furthermore, injection of 100 mU of synthetic OT to dams treated with 50 mg 2,4-D/kg bw on PPD 11, partially restored milk transfer, although they did not reach control values (Fig. 2), indicating that the blockade of milk ejection by 2,4-D is mediated, at least in part, through the inhibition of OT release.

3.4. Effect of acute or chronic 2,4-D treatment on the PRL, OT and GH levels in response of the suckling stimulus

PRL, OT and GH basal levels in the control dams were $68.33 \pm 6.29$ ng/ml, $14.56 \pm 0.73$ pg/ml and $8.34 \pm 1.38$ ng/ml, respectively and the values were not modified by any 2,4-D treatment. Both regimens of 2,4-D had similar effects on the PRL and OT response to 15 min suckling (Figs. 3 and 4). The lowest dose of 2,4-D produced a significant inhibition on PRL release, and this inhibition increased slightly with increasing doses of the herbicide (Fig. 3). The suckling-induced release of OT was not affected by the lowest doses of 2,4-D, but chronic exposure to 25 mg/kg/day (Experiment 1, Fig. 4A) or acute administration of 15 mg/kg (Experiment 2, Fig. 4B) significantly inhibited OT release, and this inhibitory effect increased at higher doses.

Circulating GH concentrations were not modified by the suckling stimulus ($9.26 \pm 0.45$ ng/ml), nor there was any effect of the 2,4-D treatments (results not shown).
Fig. 3. Effect of chronic (Panel A) or acute (Panel B) administration of increasing doses of 2,4-D on serum PRL concentrations in lactating rats after 8 h separation and 15 min of suckling on PND 13. See Section 2 for treatment schedules and further details. Each bar represents the mean serum PRL concentration ± SEM for groups of 8 dams. ***p < 0.001, **p < 0.01, *p < 0.05 compared with the control group (ANOVA followed by Student–Newman–Keuls).

3.5. Effect of acute or chronic 2,4-D treatment on hypothalamic NOS activity

Acute administration of 50 mg/kg i.p. of the herbicide had no effect (Fig. 5), but chronic exposure to 50 mg/kg/day of 2,4-D increased calcium-dependent (Fig. 5A) and independent (Fig. 5B) hypothalamic NOS activity in lactating mothers on PPD 16.

3.6. Effect of acute or chronic 2,4-D treatment on hypothalamic monoamine content

Acute or chronic treatment with 2,4-D had similar effects on ArN and AL monoamine levels, producing significant increases in DA (Tables 2 and 3). NA and HVA were not affected by the 2,4-D treatments, and DOPAC was significantly increased only in the ALs from chronically treated dams (Table 2). HVA/DA and DOPAC/DA ratios were also not modified by any treatment in ArN, while in AL both ratios were significantly diminished only by the acute treatment (Table 2).

In the ArN, 5-HT and its metabolite, 5-HIAA was decreased by both treatments and at all concentrations, but 5-HIAA levels in the AL were not modified. Since the decrease in 5-HT was much more profound than that of 5-HIAA, the 5-HIAA/5-HT ratio - which is an indicator of 5-HT turnover, was significantly increased in the ArN.

4. Discussion

The present results clearly show that acute or chronic exposure to increasing doses of 2,4-D produced a significant inhibition in milk transfer to the litter in lactating rats, that may be the main cause of the previously found decreased growth rate of pups born to mothers exposed to this herbicide (Stürtz et al., 2000, 2006).
Table 2
Effect of chronic oral administration of 2,4-D to lactating rats on the concentration of monoamines and their metabolites in dams’ arcuate nucleus (Arn) and anterior pituitary lobe (Al) on PPD 16.

<table>
<thead>
<tr>
<th>Dose of 2,4-D (mg/kg bw/day)</th>
<th>ArN</th>
<th>Control</th>
<th>15</th>
<th>25</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NA</td>
<td>55.3 ± 8.8</td>
<td>38.7 ± 8.4</td>
<td>36.2 ± 8.5</td>
<td>43.7 ± 8.9</td>
</tr>
<tr>
<td></td>
<td>DA</td>
<td>26.3 ± 1.2</td>
<td>30.5 ± 3.3</td>
<td>38.2 ± 2.0* († 44%)</td>
<td>39.9 ± 1.8** († 50%)</td>
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<tr>
<td></td>
<td>HVA</td>
<td>11.2 ± 1.5</td>
<td>19.4 ± 4.3</td>
<td>15.8 ± 3.5</td>
<td>21.4 ± 5.1</td>
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<td>HVA/DA</td>
<td>0.44 ± 0.06</td>
<td>0.63 ± 0.13</td>
<td>0.48 ± 0.10</td>
<td>0.41 ± 0.08</td>
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<td></td>
<td>DOPAC</td>
<td>8.9 ± 0.9</td>
<td>8.6 ± 2.9</td>
<td>10.8 ± 2.3</td>
<td>8.6 ± 0.9</td>
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<td>DOPAC/DA</td>
<td>0.35 ± 0.04</td>
<td>0.28 ± 0.11</td>
<td>0.24 ± 0.05</td>
<td>0.22 ± 0.02</td>
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<tr>
<td>5-HT</td>
<td>19.9 ± 1.8</td>
<td>10.3 ± 1.2* († 48%)</td>
<td>3.9 ± 2.0*** (↓ 80%)</td>
<td>3.1 ± 0.9** (↓ 84%)</td>
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<tr>
<td>5-HIAA</td>
<td>68.6 ± 3.0</td>
<td>47.8 ± 8.8* († 30%)</td>
<td>32.0 ± 1.8*** (↓ 53%)</td>
<td>32.7 ± 3.7* (↓ 52%)</td>
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<tr>
<td>5-HIAA/5-HT</td>
<td>3.9 ± 0.5</td>
<td>4.2 ± 1.9</td>
<td>17.2 ± 2.6** († 360%)</td>
<td>16.3 ± 2.0** († 323%)</td>
<td></td>
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<tr>
<td>Al</td>
<td>NA</td>
<td>204.1 ± 48.0</td>
<td>226.1 ± 53.8</td>
<td>153.2 ± 32.2</td>
<td>168.6 ± 479</td>
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<tr>
<td></td>
<td>DA</td>
<td>64.9 ± 5.5</td>
<td>124.1 ± 19.9</td>
<td>168.1 ± 8.5* († 160%)</td>
<td>217.1 ± 14.4** (‡ 234%)</td>
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<tr>
<td></td>
<td>HVA</td>
<td>40.6 ± 5.9</td>
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<td>HVA/DA</td>
<td>0.64 ± 0.09</td>
<td>0.32 ± 0.06*** († 50%)</td>
<td>0.22 ± 0.04** (↓ 66%)</td>
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<td>DOPAC</td>
<td>94.3 ± 10.1</td>
<td>140.5 ± 26.6</td>
<td>151.5 ± 3.4* († 61%)</td>
<td>160.5 ± 11.0* († 70%)</td>
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<td>DOPAC/DA</td>
<td>1.47 ± 0.25</td>
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<td>N.D.</td>
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<td>N.D.</td>
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<td>5-HIAA</td>
<td>95.9 ± 17.2</td>
<td>100.3 ± 19.5</td>
<td>90.3 ± 14.1</td>
<td>139.8 ± 22.3</td>
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</table>

Values (pmol/mg tissue) are means ± SEM of groups of 8 dams. Percentage of variation with respect to control is shown between brackets. Results were analysed by ANOVA. Where significant differences were found, means were tested using the Student–Newman–Keuls test for multiple comparisons.

* p < 0.05 compared to control values.
** p < 0.01 compared to control values.
*** p < 0.001 compared to control values.

Once lactation is established, the rate of milk secretion is adjusted to the demands of the offspring primarily by feedback mechanisms thought to be related to the amount of milk remaining in the alveoli at the termination of suckling (Neville et al., 2000). Treated 2,4-D dams were incapable to adjust the milk transfer to the litter according to pups demand even with the lowest dose evaluated or after an acute treatment.

It is known that high protein binding and active secretion of the compound would be ionized at physiological pH and for these reasons is very difficult to appeared on the milk, but these affirmation could be not true in lactating stage. The results obtained in the chronic exposure test can be influenced by the accumulation of the herbicide in the mother. This was previously demonstrated in a previous work (Stürtz et al., 2000) when the herbicide treatment was withdraw from dams, 2,4-D residues remained in stomach content of neonates, for a least after a week. It is difficult to get an accurate picture of how much and if the drug can be accumulated in the milk secretion because most of

Table 3
Effect of i.p. administration of 2,4-D on PPD 11 to lactating rats on the levels of monoamines and their metabolites in dams’ arcuate nucleus (Arn) and anterior pituitary lobe (Al) on PPD 16.

<table>
<thead>
<tr>
<th>Dose of 2,4-D (mg/kg bw)</th>
<th>Arn</th>
<th>Control</th>
<th>25</th>
<th>50</th>
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<td></td>
<td>NA</td>
<td>58.4 ± 7.4</td>
<td>33.4 ± 3.6</td>
<td>26.5 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>DA</td>
<td>22.5 ± 2.4</td>
<td>37.2 ± 6.8* († 65%)</td>
<td>37.1 ± 1.4** († 65%)</td>
</tr>
<tr>
<td></td>
<td>HVA</td>
<td>12.3 ± 1.4</td>
<td>14.7 ± 2.9</td>
<td>9.9 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>HVA/DA</td>
<td>0.45 ± 0.08</td>
<td>0.15 ± 0.02</td>
<td>0.26 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>DOPAC</td>
<td>9.3 ± 1.1</td>
<td>6.3 ± 2.9</td>
<td>5.8 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>DOPAC/DA</td>
<td>0.34 ± 0.05</td>
<td>0.19 ± 0.08</td>
<td>0.16 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>20.3 ± 1.6</td>
<td>2.0 ± 0.5* (↑ 90%)</td>
<td>2.3 ± 0.7* (↑ 89%)</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>70.3 ± 3.3</td>
<td>43.7 ± 5.3** (↑ 38%)</td>
<td>38.7 ± 3.5* (↑ 45%)</td>
</tr>
<tr>
<td></td>
<td>5-HIAA/5-HT</td>
<td>4.6 ± 0.5</td>
<td>17.2 ± 2.5** (↑ 370%)</td>
<td>17.6 ± 1.3** (↑ 380%)</td>
</tr>
<tr>
<td>Al</td>
<td>NA</td>
<td>198.9 ± 46.3</td>
<td>153.2 ± 32.2</td>
<td>168.6 ± 47.9</td>
</tr>
<tr>
<td></td>
<td>DA</td>
<td>69.4 ± 8.4</td>
<td>233.4 ± 41.1*** (↑ 336%)</td>
<td>222.2 ± 27.6** (↑ 320%)</td>
</tr>
<tr>
<td></td>
<td>HVA</td>
<td>40.7 ± 5.9</td>
<td>33.8 ± 4.6</td>
<td>26.9 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>HVA/DA</td>
<td>0.64 ± 0.09</td>
<td>0.16 ± 0.02** (↑ 75%)</td>
<td>0.14 ± 0.03*** (↑ 78%)</td>
</tr>
<tr>
<td></td>
<td>DOPAC</td>
<td>97.3 ± 11.0</td>
<td>125.0 ± 20.8</td>
<td>129.1 ± 14.4</td>
</tr>
<tr>
<td></td>
<td>DOPAC/DA</td>
<td>1.47 ± 0.25</td>
<td>0.65 ± 0.17** (↑ 56%)</td>
<td>0.64 ± 0.09*** (↑ 56%)</td>
</tr>
<tr>
<td></td>
<td>5-HT</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>5-HIAA</td>
<td>96.4 ± 15.7</td>
<td>74.5 ± 11.0</td>
<td>111.9 ± 14.1</td>
</tr>
</tbody>
</table>

Values (pmol/mg tissue) are means ± SEM of groups of 8 dams. Percentage of variation with respect to control is shown between brackets.

* p < 0.05 compared to control values.
** p < 0.01 compared to control values.
*** p < 0.001 compared to control values.
treated groups (Stürtz et al., 2006), resulting in a lower energetic acids) and a changed content of minor proteins in milk of the 2,4-D treated dams the decrease in the litter growth was not similar than the chronic exposure. These results suggest that the mechanisms involved in milk transfer to the litter may be an extremely sensitive target to the action of the herbicide and that probably the system can adjust to a limited extent the demand of milk and partially compensate the action of prolonged xenobiotic exposition. The acute suckling experiment is a more sensitive indicator of the effects of any compound on the suckling reflex, since it measures the response to suckling by hungry pups after a period of isolation. On the other hand, on Experiment 1 although the 2,4-D treatment at low doses may have impaired somewhat the suckling reflex, continued suckling by the non-satiated pups may have compensated this effect allowing the pups to obtain a normal quantity of milk, albeit perhaps, in a longer interval of time. The higher doses may have impaired more deeply the suckling response or for a longer time, resulting in significantly lower weight gain.

The diminished milk transfer to the litter observed in the treated mothers may be a direct consequence of the decreased release of PRL and OT induced by suckling after acute of chronic 2,4-D treatment, suggesting an impaired milk-ejection reflex. The effectiveness of exogenous OT given before the suckling episode in restoring the quantity of milk obtained by the pups, is a further indication of an impairment in the central effects of suckling, while the function of the mammary tissue seemed to be normal. However, incomplete voiding of the alveoli as a result of the decreased OT level may have produced a degree of milk stasis while the reduced PRL level may also have diminished milk production, since PRL is the main stimulus for milk synthesis. The probable accumulation of milk in the alveoli, consequence of the incomplete milk ejection, may also have contributed to a decrease in milk synthesis. Thus, although the OT injection increased milk ejection, it was not restored to control values, suggesting that the mammary glands of the 2,4-D treated dams accumulated less milk compared to the control dams.

It is interesting to note that while suckling induced by PRL was inhibited by all doses of 2,4-D, OT was inhibited only by the higher doses in both treatment. Thus, the reduction of milk ejection produced by the low doses of 2,4-D would be adjudicated solely to a diminished milk synthesis resulting from inadequate lactogenic stimulus. But the more profound effect observed on the pups weight gain, at higher doses of the herbicide, may be consequence of a combination of the diminished milk synthesis plus incomplete milk evacuation caused by the lowered OT secretion. Thus, suckling pups of chronic treated dams with all doses of the herbicide are malnourished as reflect in the impaired litter growth. In the pups of the acute treated dams the decrease in the litter weight was not similar than the observed in the chronic group because only the higher doses had effect in this parameter.

In a previous work, we demonstrated a reduction of 30% in the content of total lipids, fatty acids (mostly the polyunsaturated fatty acids) and a changed content of minor proteins in milk of the 2,4-D treated groups (Stürtz et al., 2006), resulting in a lower energetic content of the milk, that along with the decrease in total milk transfer to the litter may be directly responsible of the diminished growth rate of the litters from herbicide treated mothers.

A direct effect of the herbicide on the pups cannot be ruled out, but we have demonstrated a marked effect on the milk ejection reflex, that can be restored by OT treatment, and on maternal circulating PRL and OT response to suckling, indicating that an important part of the effect is exerted on the maternal organism, and any effect on the pups may only compound the problem.

Dopamine of tuberoinfundibular dopamine neurons (TIDA) origin, delivered through long portal vessels into the sinusoid capillaries of the anterior lobe, is considered the major physiological negative regulator of PRL secretion (Leong et al., 1983). It has been well demonstrated that the high and sustained levels of circulating PRL observed in lactating rats, in response to continuous suckling, are a consequence of reduced dopaminergic tone in the TIDA and tuberohypophyseal dopaminergic TTHA neurons that tonically inhibit PRL secretion (Andrews et al., 2001). We found a significant increase in DA levels in the ArN and most importantly in the AL in 2,4-D treated dams that may be directly responsible for the reduced PRL response to the suckling stimulus. The increased DA levels in the AL may be directly responsible for the reduced PRL levels and be a consequence of the increased ArN DA concentration that reflects augmented TIDA dopaminergic activity. Interestingly, 5 days after i.p. administration of one dose of 2,4-D on PPD 11 the increase on DA and DOPAC levels in ArN and AL was similar to that observed in the chronic oral treatment groups, indicating that an unique exposure to the herbicide has a long lasting effect on hypothalamic neurotransmitters.

It has also been shown that the suckling-induced PRL release in rats is mediated by the ascending serotoninergic pathways of the dorsal raphe nucleus (Fessler et al., 1984; Van de Kar and Bethea, 1982). Administration of serotoninergic neurotoxins or serotonin synthesis blockers to lactating rats reduced suckling-induced PRL release demonstrating that 5-HT facilitates suckling-induced PRL release (Jahn and Deis, 1994). Suckling results in a rapid (within 5 min) decrease in the hypothalamic concentration of serotonin and an elevation of its metabolite 5-hydroxyindoleacetic acid, simultaneous with the release of PRL (Mena et al., 1976). Receptors for serotonin are present in the anterior lobe of the pituitary gland (Calogero et al., 1993; Calogero et al., 1995), but serotonin does not stimulate PRL release in vitro (Lamberts and MacLeod, 1979; Lamberts et al., 1989), suggesting that it acts modulating PRL stimulatory or inhibitory factors, rather than through a direct action on the AL. In this study we found that both 2,4-D treatments decreased drastically 5-HT and 5-HIAA levels in the ArN, results that may have contributed to the reduced response to the suckling stimulus. Because serotonin elevates PRL more or less independently of the concentration of dopamine in the portal circulation, it has been proposed that serotonin stimulates PRL release through the action of a PRL-releasing factor, and not through modulation of the hypothalamic dopaminergic neurons (Pilotte and Porter, 1981), and dopamine infusion cannot prevent serotonin-induced PRL release (Freeman et al., 2000). Thus, chronic or acute exposure to 2,4-D would block the suckling-induced PRL release through alteration of 5-HT/DA regulatory systems.

Nitric oxide has been shown to play a regulatory role in neuroendocrine function and PRL secretion (Brann et al., 1997). Nitric oxide is synthesised from l-arginine by nitric oxide synthase (NOS) (Dawson and Snyder, 1994; Moncada et al., 1989). Within the hypothalamus, nitric oxide synthase (NADPH-diaphorase) activity is found in the paraventricular and supraoptic nuclei as well as in the lamina terminals (Bhat et al., 1995; Brett et al., 1991). A large amount of data indicates that NO has an inhibitory effect on PRL secretion (McCann et al., 1996; Aguilar et al., 1997; Dudvanski et al., 1996). The increase in NOS activity of the ArN of dams treated through the food may be due to the increased activity of dopaminergic neurons. This effect was not showed in acute treated dams perhaps they were killed on PPD 16 and the effect on NOS increase activity may be transient. This point should be evaluated in further studies. It has been shown that DA increases NOS activity in the hypothalamus (Dudvanski et al., 1996) and although NO decreases the release of DA in median basal hypothalamus it is probable that the effect seen here is overwhelmed by the increase of DA in ArN due to 2,4-D treatment.
In conclusion, we have found that the deleterious effect on litter growth of 2,4-D exposure to lactating rats is a consequence of the blockade of the suckling-induced PRL and OT release, that in turn, impair synthesis and ejection of the milk. Furthermore, this altered hormonal response to the suckling stimulus may be a result of alterations on hypothalamic and pituitary concentrations of dopamine, 5-HT and their metabolites, neurotransmitters that play key roles in the regulation of PRL release. Thus, the presence of elevated amounts of 2,4-D in sprayed fields can pose a severe threat for the reproductive success of wild life, livestock, domestic animals and even for human beings exposed to them.

Conflict of interest statement
None.

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References


Bortolozzi, A., Duffard, R., Evangelista de Duffard, A.M., 2003. Asymmetrical develop-
ment of the monoamine system in 2,4-dichlorophenoxyacetic acid treated rats. Neurotoxcol. Teratom. 24, 149–157.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of micro-
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