LONG-TERM EFFECT OF DEVELOPMENTAL EXPOSURE TO CHLORPYRIFOS ON HYPOTHALAMIC OXITOCIN AND VASOPRESSIN LEVELS IN MICE

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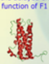
ABSTRACT

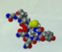
Exposure to endocrine disrupters can permanently influence the foetal programming of the several body systems through the alteration of signalling networks. The hypothalamus-hypophysis-gonad (HHG) axis is a complex system which influences the programming of several organs and their functions through feed-back mechanisms as well as different signalling proteins such as prolactin, arginine vasopressin and oxitocin. Organophosphate insecticides (e.g. Chlorpyrifos, CPF) – still widely used in agriculture and against household pests - receive an increasing consideration for their interference with endocrine function, possibly by interacting with HHG axis.
We present here preliminary data on the delayed effects of pre- and/or postnatal exposure to CPF on hypothalamic function of F1 female and male mice, at dose levels devoid of maternal toxicity. .
Pregnant CD1 mice (10/group) were treated po with 0 (vehicle corn oil), 3, 6 mg/kg/bw/day of CPF on gestational days 15-18. Following delivery, 10 newborns/sex/group (F1) were treated subcutaneously on post-natal days (PND) 11-14 with: 0, 1, 3 mg/kg/bw/day of CPF. Protein expression of prolactin, arginine vasopressin and oxytocin were evaluated by Western Blot or ELISA analysis, in the hypothalamus of F1 males and females sacrificed at adulthood (PND 150).
In male mice, oxytocin and vasopressin levels resulted inversely proportional to each other with respect to CPF treatments. Female mice resulted affected to a lesser extent, being generally more susceptible to pre-natal treaments. Prolactin levels were higher in female mice that received 3 mg/kg/bw/day as prenatal treatment; the assay on male mice is still in progress.
Our preliminary data suggest that prenatal exposure to CPF may alters the expression of major signalling proteins of HHG axis and that such effects are still evident at adulthood. The consequences of such altered endocrine programming deserve further investigation, also with respect to the reported changes in maternal and social behaviour of CPF-exposed females and males.

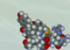
INTRODUCTION

Long-term exposure to food and environmental contaminants at doses apparently without evident morphological effects might represent a major risk factor for children's health since they can permanently affect different target organs/systems after in utero exposure. Chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothionate - CPF) is a broad-spectrum organophosphate insecticide, commonly used also in households and community sites until restrictions on its residential use were recently placed in industrialized Countries (1, 2). CPF oxon is the main metabolite of CPF which prevents acetylcholine degradation, thus acting as cholinesterase inhibitor. However, there are suggestions that CPF may impair neurobehavioural development through mechanisms other than cholinesterase inhibition (3). The HHG) axis, which integrates inputs to and outputs from the nervous and reproductive systems, is functionally and anatomically situated to mediate developmental effects of environmental toxicants, particularly those that are endocrine-disrupting chemicals (EDCs) (4). Both the in vitro and in vivo findings support the concept of CPF as an EDC (5, 6). In vivo studies suggest that CPF causes significant alterations in GnRH mRNA levels in female rats. Several possible functional targets of EDCs may be videntified in the HHG axis. In the hypothalamus are located prolactinergic neuronal cell bodies which sent axonal terminals to all the part of the brain. Evidence suggests that hypothalamic prolactin (PRL) synthesis is independent from anterior pituitary and that this function is involved in the modulation of neuronal activity and behaviour (7).
Oxytocin (OXY) is a very abundant neuropeptide of nine amino acids exerting a wide spectrum of central and peripheral effects as neurohormone, neurotransmitter, or neuromodulator. OXY gene is predominantly expressed in magnocellular neurons in the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei. OXY is secreted both in the general circulation, in the neurohypophysis as well as in the CNS. OXY receptors have also been identified in other tissues, including the kidney, heart, thymus, pancreas and adipocytes. Besides its stimulatory action of uterine smooth muscle during labor and mammary gland during lactation, OXY is recognized as having endocrine and paracrine roles in mediating some reproductive and social behaviours, learning and memory in a gender-related manner (8).
The arginine vasopressin (AVP) neuropeptide is also synthesised in PVN and SON and share a similar structure with OXY, being composed of nine amino acids but differing in only two residues. Therefore it exists a functional cross-reactivity between them. AVP controls, even at low doses, the resorption of water in the kidneys and the osmotic content of blood. At high doses, AVP causes contraction of arteriles and capillaries producing localized increase in blood pressure. Recent evidence indicate that AVP is regulated by steroids and is involved in modulation of social behaviour in rodents (9).
Behavioral and neural alterations have been extensively documented in rats developmentally exposed to CPF following subtoxic exposures notably in absence of significant brain AChE inhibition (10). As a whole, in rat studies most of the behavioural effects appear to be gender-selective and dependent on time of CPF administration, as different critical phases of sensitivity have been identified for the effects of this agent on the developing brain: the late gestation (gestational days GDs 17-20) and the neonatal stage (postnatal days PNDs 1-4 and 11-14), characterized by different CNS maturational events (10).
We present here preliminary data on the delayed effects of pre- and/or postnatal exposure to CPF at dose levels devoid of maternal toxicity on hypothalamic function of F1 female and male mice.


Schematic representation of HHG axis


3D structure of PRL


Spacefill model of OXY


Spacefill model of AVP

MATERIALS & METHODS

Animals and treatments

Pregnant CD1 mice (10/group) were treated with 0 (vehicle corn oil), 3 or 6 mg/kg/bw/day of CPF on gestational day (GD) 15-18. Before, during and at the end of treatment, dam body weight gain and food consumption were registered After delivery, litters were reduced to 5 pups for each sex. Three subgroups of newborns were treated subcutaneously on PND 11-14 with: 0 (corn oil vehicle), 1 or 3 mg/kg/bw/day of CPF to have 9 final experimental groups (see Tab. 1).

Tab 1

M0P0	M0P1	M0P3	M3P0	M3P1	M3P3	M6P0	M6P1	M6P3

M = dam treatment;
P = newborns' post-natal treatment;
0 = 0 mg/kg/bw/day; 1 = 1 mg/kg/bw/day; 3 = 3 mg/kg/bw/day; 6 = 6 mg/kg/bw/day
Five males and five females for group.

Pups were weaned on PND 23, and offspring of both sexes assessed for a number of behavioural responses which are the subject of two other studies (see Ricceri et al, 2006, Tox Sci; Venerosi et al., 2006, Neurotoxicol Teratol). At PND 150 F1 animals were sacrificed, hypothalami were excised and frozen for protein analysis.

Samples preparation

Mice hypotalami were lysed in 100 µl Lysis Buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 5 mM EDTA, supplemented with 1mM phenylmethylsulfonylfluoride [PMSF]) and homogenised by means of a 2 ml sterile siringe. Following 1h incubation on ice, lysates were centrifuged at 13.000 rpm, 4 °C, for 20 min, then two supernatants per treatment were pooled together to avoid individual variability. Protein concentration in each sample was determined by the BCA method using bovine serum albumin (BSA) as standard.

Western Blot analysis

50 µg of total extract proteins per lane were separated in 12% SDS-PAGE using the pre-stained SeeBlue® molecular markers (Invitrogen). The gel was then electoblotted onto a PVDF membrane (Bio-Rad) by a semi-dry Trans-Blot (SciePlus). The blotted membranes were then washed with phosphate buffered saline (PBS) and blocked O/N with a 5% non-fat dry milk solution. After washing with PBS containing 0,1% Tween 20 (PBST) the membranes were incubated 1,5h at room temperature (RT) with an anti-prolactin (Santa Cruz) or an anti-vasopressin antibody (Chemicon, <1% cross-reactivity with oxytocin), in PBS containing 5% non-fat dry milk. Following washing with PBST the membranes were incubated 1h RT with the appropriate secondary antibody HRP conjugated (Santa Cruz) diluted in PBS containing 5% non-fat dry milk. The blots were visualised using a chemiluminescent detection system (Santa Cruz) and exposed to a x-ray film (Kodak). Bands desitometry was performed with the Quantity-One software (Bio-Rad).

ELISA analysis

For each treated pool a solution of 50 µg/ml of total extract proteins in Coating buffer (0,1M $Na_2CO_3/NaHCO_3$) was allowed to coat on a 96 flat bottomed multiwell plate (100 µl/well) for 1h at 37 °C then O/N at 4 °C. After washing with PBS the wells were blocked 2h RT with a 5% non-fat dry milk solution. Plates were then washed with PBST and incubated for 1,5h RT with an anti-oxytocin antibody (Chemicon) diluted in PBS containing 5% non-fat dry milk (100 µl/well). Following washing with PBST plates were incubated 1h RT with a secondary HRP conjugated antibody (Santa Cruz) diluted in PBS containing 5% non-fat dry milk 100 µl/well). Detection was performed with the TBM system (Vectastain) reading absorbance at 450 nm with a Victor 3 Multilabel Reader (Perkin Elmer).

RESULTS

OXYTOCIN LEVELS

Evaluation of OXY levels apparently show a sexual dimorphic pattern of expression. In fact, in male mice OXY levels seem to be affected in a dose-related way. In female mice, OXY levels are generally lower than those in the sibling male mice, and such effect is associated essentially with prenatal treatment, whether it was followed or not with postnatal treatment (Fig. 1A).

VASOPRESSIN LEVELS

AVP levels seem to show an inverse sexually dimorphic pattern of expression. Hypothalami of treated male mice present decreasing amount of AVP in an inverse dose related way. Female treated mice showed a slightly higher expression of AVP which increased in the higher post-natal doses (Fig. 1B).

A B

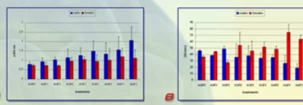


Fig. 1 - A) Mean values for OXY levels derived by three indipendent replica of ELISA assays. A main effect of prenatal treatment [F (2, 8) = 5.04, p=0.038] indicates that prenatal CPF significantly increases OXY levels in both males and females. A main effect of postnatal treatment is also evident [F (2, 8) = 7.00, p=0.017] indicating that postnatal CPF significantly increases OXY levels; interestingly this postnatal CPF effect is more pronounced in males [interaction sex x postnatal treatment F (2, 8) = 12.23, p=0.003]. B) Mean values for AVP levels derived by two indipendent replica of WB assays. These data show a tendency for a differential effect of prenatal CPF treatment in the two sexes (AVP increases in females and decreases in males). This effect, however is not statistically significant because of limited sample size.

PROLACTIN LEVELS

Hypothalami of female mice present a peculiar pattern for PRL expression, CPF apparently affecting the intermediate dose level of prenatal treatment (3 mg/kg/bw), with less evident changes for the other treatments. The assay on male mice are still in progress (Fig. 2).

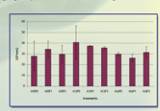


Fig. 2 - Mean values for PRL levels in females derived by two indipendent replica of WB assays. This effect is not statistically significant because of limited sample size.

DISCUSSION

Pre-natal and/or post-natal exposure to EDCs, is of particular concern for long-term effects on CNS functional development, including social behaviour, learning and memory, , particularly because of their interactions with the HHG axis. We have hypothesised that the pesticide CPF could affect neuroendocrine modulation through a mechanism other than the established cholinesterase inhibition. To assess such a mechanism, we investigated the hypothalamic expression of three major neuromodulators, namely PRL, AVP and OXY, following pre- and/or post-natal exposure of CD1 mice. Effects on the three neuromodulators were detected in fully adult (5 month-old) mice.

- Protein analysis shows patterns of expression which seems to be gender-related. In particular, in male mice OXY increases and AVP decreases in association with the dose level; in females AVP increases whereas OXY increases, too, but only in relation with the pre-natal treatment.
- Male mice seem to be more susceptible to post-natal exposure in comparison to females which appear more sensitive to pre-natal treatment.

This pattern of effects is paralleled by alterations in sex-dimorphic behaviours in CPF exposed offspring, involving social/affiliative responses and maternal propensity in females, and agonistic behaviour in adult males (11, 12). In particular CPF exposed male mice express higher levels of aggression towards an unfamiliar conspecific, and such finding is in line with the well Known proaggressive role of hypothalamic oxitocin in male rodents.
Such preliminary results could suggest a different, gender-related window of susceptibility for the functional development of HHG axis in mice. Moreover, the possible interference of CPF in the cross-talk between OXY and AVP regulatory pathways is suggested. Our preliminary results indicate the importance of further studies on the possible EDC interactions with the HHG axis and their functional implications.

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