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ORIGINAL

Permanent sexual and regional noradrenergic and dopaminergic systems impairment after prenatal and postnatal exposure to chlordimeform

Alteraciones permanentes de los sistemas noradrenérgicos y dopaminérgicos de forma región y sexo dependiente tras exposición prenatal y postnatal al clordimeformo

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Abstract

Introduction: Formamidines pesticides have been described to induce permanent effects on development of monoaminergic neurotransmitter systems. The mechanisms that induce these effects are not known but it has been suggested that these effects could be related to monoamino oxidase (MAO) inhibition. Chlordimeform is a formamidine pesticide, which is a very weak inhibitor of MAO although it has been described to induce permanent and sex dependent alterations of serotoninergic system.
Objectives and methods: In order to confirm that formamidines induce permanent alterations of monoaminergic neurotransmitter systems regardless of MAO inhibition, the effects of maternal exposure to chlordimeform (5 mg/kg bw, orally) on brain region dopamine and noradrenaline levels of male and female offspring rats at 60 days of age were evaluated. The results showed that chlordimeform induced a significant decrease of noradrenaline and dopamine levels in the prefrontal cortex and striatum and of dopamine levels in the hippocampus, showing an interaction by sex for these regions.
Results: Chlordimeform also caused a decrease of DOPAC levels in the striatum and of MHPG and HVA metabolites levels in the prefrontal cortex and striatum. Moreover, it induced an increase in the content of metabolites DOPAC and HVA in the hippocampus and an increase in the metabolite content of DOPAC in the striatum. Lastly, it increased the turnover of DA in the hippocampus and striatum and decreased the turnover of NA and DA in frontal cortex, as well as the NA in striatum.
Conclusions: The present findings indicate that maternal exposure to chlordimeform altered dopaminergic and noradrenergic neurochemistry in their offspring in a region and sex dependent way, and those variations confirm that other mechanisms different from MAO inhibition are implicated.

Keywords: Chlordimeform; formamidines; neurodevelopmental toxicity; dopamine; noradrenaline; rats; human risk assessment

Resumen

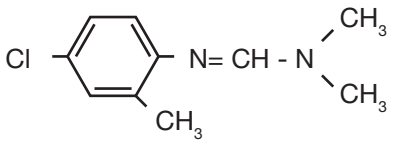
Introducción: Se ha descrito que los pesticidas formamidínicos inducen efectos permanentes en el desarrollo de los sistemas de neurotransmisores monoaminérgicos. Los mecanismos por los que se inducen estos efectos no se conocen, pero se ha sugerido que podrían estar relacionados con la inhibición de la monoamino oxidasa (MAO). El clordimeformo, es un pesticida formamidínico, del que se han descrito que induce una alteración permanente del sistema serotoninérgico región y sexo dependiente, aunque es un inhibidor muy débil de la MAO.
Objetivos y métodos: Con el objetivo de confirmar que las formamidinas produce alteraciones permanentes de los neurotransmisores monoaminérgicos independientemente de la inhibición de la MAO, se evaluaron los efectos sobre los niveles de dopamina y serotonina en regiones cerebrales de ratas macho y hembra a los 60 días de edad tras la exposición maternal al clordimeformo (5 mg/kg de peso corporal).
Resultados: El clordimeformo indujo una disminución significativa de los niveles de noradrenalina y dopamina en las regiones cerebrales corteza frontal, cuerpo estriado, así como de la dopamina en el hipocampo mostrando una interacción por sexo en esta regiones. El clordimeformo además, originó un descenso de los metabolitos MHPG y HVA en corteza frontal y cuerpo estriado y del metabolito DOPAC en el cuerpo estriado. También, indujo un aumento en el contenido de los metabolitos DOPAC y HVA en hipocampo y un aumento del contenido de DOPAC en el cuerpo estriado. Por ultimo aumentó la tasa de recambio de DA en el hipocampo y cuerpo estriado y disminuyó la tasa de recambio de la NA y DA en corteza frontal y así como de la NA en cuerpo estriado.
Conclusiones: Los presentes resultados indican que las formamidinas y en particular el clordimeformo, inducen, tras la exposición maternal, una alteración permanente de los sistemas dopaminérgico y noradrenérgico de forma región y sexo dependiente en la descendencia, lo cual confina que estas alteraciones se deben a mecanismos distintos de la inhibición de la MAO.

Palabras clave: Clordimeformo, formamidinas, neurotoxicidad en el desarrollo, dopamina, noradrenalina, ratas, evaluación del riesgo para el hombre

Introduction

Pesticides pose a growing risk to health, with a specific concern about the possible permanent effects that these compounds may have on the development of organisms. Formamidine pesticides has been reported to induce permanent alteration of brain development. In this regard, it has been described for the formamidine compound amitraz, the induction of permanent alterations on the development of central nervous system (CNS) such as those that affect monoamine neurotransmitter systems[1]. Moreover, chlordimeform [N2-(4-chloro-o-tolyl)-N1.N1-dimethylformamidine] (**Figure 1**), which is another member of formamidines family, it has been also reported that induce permanent alterations of serotoninergic system[2]. The mechanism by which these effects occur is not known.

Figure 1: Chlodimeform chemical structure (C10H13Cl N2).



On the other hand, monoamine oxidase (MAO) inhibition was among the first biochemical actions of the formamidines that were reported[3-4]. Thus, aminergic mechanism of action of chlordimeform was quickly postulated and adopted because neuronal MAO participates in metabolic inactivation of biogenic monoamines which include the neurotransmitters serotonin, norepinephrine, and dopamine. In addition, chlordimeform is an antagonist of reserpine effects[5], alters prostaglandin synthesis[6], has $\alpha 2$ receptor agonist properties[7], and is an endocrine disruptor[8].

Currently it is assumed that the monoaminergic neurotransmitters play a role during development, defined as "morphogenetic"[9-12]. Any change in the levels of catecholamines during development could have a profound effect on brain development, both structural and functional[13]. In this sense, it has been suggested as a possible mechanism of action the inhibition of MAO which may alter the levels of monoaminergic neurotransmitters, although other mechanisms as endocrine disruption on sex hormones that control the expression of enzymes that catalyze the synthesis and metabolism of the monoamine neurotransmitters cannot be excluded[2].

According to all exposed above, we performed a study to establish if maternal exposure to formamidines during gestation and lactation induces permanent alterations on dopaminergic and noradrenergic systems in adult age. Chlordimeform was chosen because it is the most representative compound in its group which presents a very low inhibition of MAO, allowing us to study more clearly whether the permanent changes observed on levels of these neurotransmitters are due to an alteration of the enzymes that catalyze the synthesis and metabolism of these neurotransmitter rather than inhibition of MAO.

This work focuses its interest in providing new data of formamidines induced neurotoxicity during nervous system development, because new compounds of this family are being developed with therapeutic applications for which these effects are not considered in their risk assessment, which poses a potential health hazard.

Materials and methods

Biological material

All experiments were performed in accordance with European Union guidelines (2003/65/CE) and Spanish regulations (BOE 67/8509-12, 1988) regarding the use of laboratory animals. Six pregnant Wistar rats were housed individually in polycarbonate cages and were assigned randomly to two experimental groups: a chlordimeform treatment group (n = 3) and a control group (n = 3).

Test Chemical and Treatment

Chlordimeform (Sigma, Madrid, Spain) was dissolved in corn oil to provide fast and complete absorption and was administered orally by gavage in a volume of 2 mg/ml. The animals received daily chlordimeform at the dose of 5 mg/kg on days 6 to 21 of pregnancy (GD 6-21) and on days 1 to 10 of lactation (PN 1-10). Control dams received vehicle (corn oil 2.5 ml/kg) on the same schedules. Dose of chlordimeform was selected based on a previous preliminary study that indicated this dose was the higher one that did not cause weight loss or mortality, any reduction of food or water intake as well as did not induce haematological modifications of other clinical histopathological signs of overt toxicity. Moreover we did not see any changes in suckling of maternal caretaking. None of the prenatal or postnatal treatment evoked a significant change in weight of any of the brain regions on PN 60 (data not shown).

Dams were examined daily throughout the gestation and lactation periods for mortality, general appearance and behavior. The maternal body weights were measured on GD 1, GD 5, GD 6, GD 15 and GD 20. Food and water consumption during pregnancy, length of gestation, litter size and sex ratio were also assessed.

On PN1, all litters were examined externally, sexed and weighed. Litters were organized in groups of twelve pups, six males and six females. Litters were weighed at PN 1, PN 7, PN14 and PN 21. The offspring were weaned on lactation day 21 and were maintained in appropriate conditions, housed individually and without any treatment with full access to food and water until adult age. The study was organized in treated groups of six males and six females randomly selected respectively from the dams'

litters exposed to chlordimeform, and control groups of six males and six female's pups randomly selected respectively from the control dams' litters.

At PN 60, male and female rats from control and treated groups (pups from control dams, and pups from dams exposed to chlordimeform, respectively) were sacrificed by decapitation. The brain was removed quickly and the hypothalamus, midbrain, medulla oblongata, cerebellum, brainstem, hippocampus, striatum and prefrontal cortex were rapidly dissected out at 4ºC[14]. Tissues were rapidly weighed and stored at -80 ºC until analysis. All data were collected by experimenters blind to the treatment condition of the offspring.

Determination of monoamine levels

Following sample collections, 300-800 µl of 0.4 M HClO4 containing 0.1% (w/v) $Na_2S_2O_5$ was added to the tissues, and the mixture was homogenized by sonication before neurochemical evaluation was performed. The homogenates were centrifuged for 15 min at 20000 g at 4ºC and aliquots of supernatants were taken for analysis of norepinephrine (NE), dopamine (DA) and its metabolites [3,4-hydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA)] using a high performance liquid chromatography (HPLC) technique with electrochemical detection[15,2]. Also, aliquots of supernatants were taken for analysis of the norepinephrine metabolite [3-metoxy-4-hydroxyphenylethyleneglycol (MHPG)] by HPLC with fluorimetric detection[16,2]. Volumes of 200-300 µl of the supernatants (in 0.4 M HClO4) were treated for 3 min at 100 ºC in a water bath. The samples were then cooled and 30-45 µl of 2 M NaOH were added (final pH: ca. 1.5) and aliquots were injected into a reversed phase HPLC system. For the analysis of the catecholamines NA, DA, DOPAC and HVA, the mobile phase consisted of 0.1 M $Na_2HPO_4.2H_2O$, 0.1 M citric acid (pH 3.5), 1.6 mM octane sulphonic acid, 0.9 mM EDTA and 10% (v/v) methanol. Elution was performed at a flow rate of 1 ml/min and the working electrode potential was set at 0.85 V for catecholamines. For the analysis of the norepinephrine metabolite (MHPG), the mobile phase consisted of 0.06 M $Na_2HPO_4.2H_2O$, 0.03 M citric acid and 6% (v/v) methanol. Elution was performed at a flow rate of 1.5 ml/min. Excitation and emission wavelengths of the detector were 275 and 315 nm, respectively.

Peak areas in the sample chromatograms were quantitated by external standard technique using solutions of the catecholamines (NE, DA, DOPAC and HVA), and norepinephrine metabolite (MPHG). DA and NA turnover was calculated as ratio of metabolite to neurotransmitter.

Data analysis

Statistical analysis of data was performed using a Statgraphics software, version Plus 4.1 for windows. Values are expressed as mean ± S.E.M. obtained from 12 animals, six males and six females, in each group (control and treated groups). For values combined for males and females, a two-way ANOVA with treatment × sex interaction was the initial test used. Where a significant treatment × sex interaction was detected, a separate Student's t test was carried out for each sex. The results were considered significant at $P<0.05$. Results significantly different from controls are also presented as change from control (%).

Results

Maternal and offspring body weight, physical and general activity development were unaffected by the exposure of dams to chlordimeform (5 mg/kg bw orally on days 6 to 21 of pregnancy and 1 to 10 of lactation).

Brain tissues levels of dopamine, noradrenaline, its metabolite and the turnover in rat pups at PN 60 are presented in **Tables IA** and **IIA**. In the hypothalamus, midbrain, cerebellum, medulla oblongata and brainstem the levels of DA, NA, DOPAC, HVA and MHPG, and the DA and NA turnover were not modified by dam exposure to chlordimeform in males and females rat pups at PN 60. In male and female offspring, chlordimeform induced a significant decrease of noradrenaline and dopamine content in the prefrontal cortex and striatum and of dopamine content in the hippocampus compared to control animals (**Tables IB** and **IIB**). Chlordimeform also caused a decrease in the metabolites levels of DOPAC in the striatum and MHPG and HVA in the prefrontal cortex and striatum in males and females offspring (**Tables IB** and **IIB**). Moreover, it induced an increase in the content of metabolites DOPAC and HVA in the hippocampus and an increase in the metabolite content of DOPAC in the striatum in males and females offspring (**Tables IB** and **IIB**). Lastly, it increased the turnover rate of DA in the hippocampus and striatum and decreased the turnover rate of NA and DA in frontal cortex, as well as the NA in striatum in males and females offspring (**Tables IB** and **IIB**). Chlordimeform displayed in the striatum for dopamine content, in the prefrontal cortex and hippocampus for dopamine, its metabolites and turnover, and in the striatum and prefrontal cortex for noradrenaline, its metabolite and turnover a significant sex interaction with the treatment effect (**Tables IB** and **IIB**).

Discussion

The present study shows that prenatal and postnatal exposure to chlordimeform (5 mg/kg bw orally on days 6 to 21 of pregnancy and 1 to 10 of lactation) was not able to induce maternal toxicity since during pregnancy maternal weight gain of treated rats was not modified. However, chlordimeform administered during pregnancy and lactation leads to permanent alterations of the dopaminergic and noradrenergic systems in a sex and region dependent way at 60 days of age in rats. Chlordimeform affected the content of DA and NA only in the regions frontal

Table IA: PFC: prefrontal cortex. Other tissue values were not evaluated because of the lack of treatment × sex interactions.
Values are mean ± S.E.M.; control animals (n= 6 males, n= 6 females); treated group (n= 6 males, n= 6 females).
Statistical significance is reported for the ***P*<0.01 and ****P*<0.001 levels compared with the control group within each sex as determined by one-way ANOVA, followed by the Student´s *t* test.
[a] Percentage change from control values.

Tissue	DA (ng/g)		DOPAC (ng/g)		HVA (ng/g)		(DOPAC+HVA)/DA	
	Control group	Treated group (pups from treated dams)	Control group	Treated group (pups from treated dams)	Control group	Treated group (pups from treated dams)	Control group	Treated group (pups from treated dams)
HT	1296,34 ± 8,02	1306,23 ± 10,47	110,24 ± 1,71	111,28 ± 3,13	37,67 ± 0,89	37,92 ± 1,18	0,11 ± 0,00	0,1 ± 0,00
MB	1262,29 ± 27,37	1276,79 ± 26,49	60,22 ± 1,31	60,84 ± 0,84	34,00 ± 0,92	34,33 ± 1,09	0,07 ± 0,00	0,07 ± 0,00
CB	91,86 ± 0,69	92,34 ± 0,84	22,18 ± 0,38	22,22 ± 0,49	11,22 ± 0,30	11,24 ± 0,27	0,36 ± 0,01	0,36 ± 0,00
MO	304,44 ± 2,47	306,42 ± 4,52	21,19 ± 0,30	21,32 ± 0,32	11,03 ± 0,23	11,08 ± 0,22	0,11 ± 0,00	0,11 ± 0,00
BS	464,55 ± 4,26	469,47 ± 2,81	10,88 ± 0,26	10,94 ± 0,31	9,67 ± 0,08	9,73 ± 0,14	0,04 ± 0,00	0,04 ± 0,00
PFC	495,56 ± 5,25	[b]378,02 ± 22,96	46,03 ± 0,43	[b]33,54 ± 1,74	31,25 ± 1,39	[b]22,52 ± 0,29	0,16 ± 0,00	[b]0,15 ± 0,00
ST	7234,48 ± 23,25	[b]6337,99 ± 43,89	821,85 ± 8,19	1118,99 ± 8,16***, [a](36,15%)	550,04 ± 4,49	456,62 ± 4,38***, [a](-16,98%)	0,19 ± 0,00	0,25 ± 0,00***, [a](31,12%)
HC	514,42 ± 13,75	[b]412,91± 28,34	9,55 ± 0,18	[b]11,68 ± 0,41	4,69 ± 0,08	[b]7,55 ± 0,40	0,03 ± 0,00	[b]0,05 ± 0,00

HT: hypothalamus; **MB:** midbrain; **CB:** cerebellum; **MO:** medulla oblongata; **BS:** brainstem; **PFC:** prefrontal cortex; **ST:** striatum; **HC:** hippocampus.
Data represent means ± S.E.M. with values for males and females combined (n=12: 6 males + 6 females).
Statistical significance is reported for the **P*<0.05, ***P*<0.01 and ****P*<0.001 levels compared with the control group.
[a] Percentage change from control values.
[b] Significant treatment × sex interaction.

Table IB: Statistical analysis for tissue values with significant treatment × sex interaction.

Tissue		DA (ng/g)		DOPAC (ng/g)		HVA (ng/g)		(DOPAC+HVA)/DA	
		Control group	Treated group (pups from treated dams)	Control group	Treated group (pups from treated dams)	Control group	Treated group (pups from treated dams)	Control group	Treated group (pups from treated dams)
PFC	**Males**	504,63 ± 3,24	431,46 ± 2,45***, [a](-14,50%)	46,01 ± 0,47	37,58 ± 0,31***, [a](-18,33%)	28,17 ± 0,23	22,83 ± 0,32***, [a](-18,98%)	0,15 ± 0,001	0,14 ± 0,001***, [a](-4,77%)
	Females	486,49 ± 4,14	324,59 ± 3,43***, [a](-33,28%)	46,05 ± 0,44	29,51 ± 0,22***, [a](-35,92%)	34,32 ± 0,63	22,21 ± 0,20***, [a](-35,28%)	0,17 ± 0,00	0,16 ± 0,00*, [a](-3,53%)
ST	**Males**	7197,85 ± 18,59	6237,08 ± 11,46***, [a](-13,35%)	–	–	–	–	–	–
	Females	7271,10 ± 17,51	6438,90 ± 5,82***, [a](-11,45%)	–	–	–	–	–	–
HC	**Males**	483,85 ± 3,20	347,08 ± 4,26***, [a](-28,27%)	9,82 ± 0,08	10,89 ± 0,13***, [a](10,88%)	4,79 ± 0,09	8,47 ± 0,10***, [a](76,92%)	0,03 ± 0,00	0,06 ± 0,00*, [a](84,76%)
		545,00 ± 5,63	478,73 ± 3,91***, [a](-12,16%)	9,28 ± 0,19	12,47 ± 0,32***, [a](34,32%)	4,59 ± 0,06	6,64 ± 0,10***, [a](44,55%)	0,03 ± 0,00	0,04 ± 0,00***, [a](56,72%)

PFC: prefrontal cortex. Other tissue values were not evaluated because of the lack of treatment × sex interactions.
Values are mean ± S.E.M.; control animals (n= 6 males, n= 6 females); treated group (n= 6 males, n= 6 females).
Statistical significance is reported for the ***P*<0.01 and ****P*<0.001 levels compared with the control group within each sex as determined by one-way ANOVA, followed by the Student´s *t* test.
[a] Percentage change from control values.

cortex, hippocampus and striatum displaying a sex interaction with the treatment effect. The effects observed in our study included a significant decrease of noradrenaline and dopamine contents in the prefrontal cortex and striatum and of dopamine content in the hippocampus of male and female offspring. Chlordimeform also caused a decrease of DOPAC levels in the striatum and of MHPG and HVA metabolites levels in the prefrontal cortex and striatum, although it induced an increase in the content of metabolites DOPAC and HVA in the hippocampus and an increase in the metabolite content of DOPAC in the striatum of male and female offspring. Lastly, it increased the turnover of DA in the hippocampus and striatum and decreased the turnover of NA and DA in frontal cortex, as well as the NA in striatum of male and female offspring.

Developmental neurotoxicity involves alterations in behavior, neurohistology, neurochemistry and/or gross dysmorphology of central nervous system occurring in the offspring, as a result of chemical exposure of the mother during pregnancy or lactation. The mechanism through which these permanent effects on monoaminergic systems take place is unknown, but monoamine neurotransmitters, such as 5-HT, DA and NA regulate brain deve-

Table IIA: Tissue NA and MHPGconcentrations in male and female rat pups observed at 60 days of age after the exposure of dams to chlordimeform (5 mg/kg bw orally on days 6 to 21 of pregnancy and 1 to 10 of lactation).

Tissue	NA (ng/g)		MHPG (ng/g)		MHPG/NA	
	Control group	Treated group (pups from treated dams)	Control group	Treated group (pups from treated dams)	Control group	Treated group (pups from treated dams)
HT	1338,98 ± 10,80	1355,06 ± 17,17	48,47± 0,75	49,06 ± 0,79	0,04 ± 0,00	0,04 ± 0,00
MB	579,97± 15,72	587,54± 20,84	45,00± 0,67	45,50± 0,62	0,08± 0,00	0,08± 0,00
CB	232,96± 3,39	235,09± 5,97	24,40± 0,55	24,54± 0,57	0,10± 0,00	0,10± 0,00
MO	244,22± 2,77	244,00± 3,98	28,45± 0,25	28,67± 0,39	0,12± 0,00	0,12± 0,00
BS	420,66± 8,99	426,74± 7,01	23,51± 0,33	23,84± 0,43	0,06± 0,00	0,06± 0,00
PFC	234,37± 4,08	[b]180,77± 6,69	52,74± 0,45	[b]37,64± 1,51	0,23± 0,00	[b]0,21± 0,00
ST	165,73± 3,56	[b]139,80± 6,09	81,01± 0,68	[b]59,69± 3,66	0,49± 0,01	[b]0,43± 0,01
HC	242,26± 7,86	245,86± 9,21	34,78± 0,30	35,04± 0,30	0,14± 0,00	0,14± 0,01

HT: hypothalamus; **MB:** midbrain; **CB:** cerebellum; **MO:** medulla oblongata; **BS:** brainstem; **PFC:** prefrontal cortex; **ST:** striatum; **HC:** hippocampus.
Data represent means ± S.E.M. with values for males and females combined (n=12: 6 males + 6 females).
Statistical significance is reported for the *P<0.05, **P<0.01 and ***P<0.001 levels compared with the control group.
[a] Percentage change from control values.
[b] Significant treatment × sex interaction.

Table IIB: Statistical analysis for tissue values with significant treatment × sex interaction

Tissue		NA (ng/g)		MHPG (ng/g)		MHPG/NA	
		Control group	Treated group (pups from treated dams)	Control group	Treated group (pups from treated dams)	Control group	Treated group (pups from treated dams)
PFC	Males	243,71 ± 1,15	196,29 ± 1,05***, [a](-19,46%)	52,43 ± 0,33	41,13 ± 0,16***, [a](-21,55%)	0,22 ± 0,001	0,21 ± 0,001*, [a](-2,59%)
	Females	225,04 ± 0,60	165,25 ± 0,95***, [a](-26,57%)	53,05 ± 0,54	34,15 ± 0,33***, [a](-35,63%)	0,24 ± 0,003	0,21 ± 0,003***, [a](-12,33%)
ST	Males	173,40 ± 1,19	154,01 ± 0,80***, [a](-11,18%)	81,98 ± 0,68	68,21 ± 0,60***, [a](-16,80%)	0,47 ± 0,01	0,44± 0,00**, [a](-6,35%)
	Females	158,07 ± 1,71	125,59 ± 0,39***, [a](-20,54%)	80,04 ± 0,40	51,18 ± 0,34***, [a](-36,06%)	0,51 ± 0,01	0,41 ± 0,00***, [a](-19,58%)

PFC: *prefrontal cortex. Other tissue values were not evaluated because of the lack of treatment × sex interactions.*
Values are mean ± S.E.M.; control animals (n= 6 males, n= 6 females); treated group (n= 6 males, n= 6 females).
*Statistical significance is reported for the **P<0.01 and ***P<0.001 levels compared with the control group within each sex as determined by one-way ANOVA, followed by the Student's t test.*
[a] Percentage change from control values.

lopment prior to assuming their roles as transmitters in the mature brain[17-19], thus any circumstance that affects these neurotransmitters in the developing brain can alter the final structure and function of that brain. Since the endogenous levels of DA and NA are highly regulated by MAO, any change in this enzyme can profoundly affect the developing brain. In this regard, it has been reported that gestational exposure to MAO inhibitors clorgyline and deprenyl produces in offspring at 30 days of age, a significant reduction of serotonergic innervation particularly in the cerebral cortex[20], but not in the dopaminergic and noradrenergic innervation which suggests that besides MAO inhibition other mechanism should be implicated in the alteration observed. On the other hand, amitraz, which is a potent MAO inhibitor[3], has been shown to induce permanent alterations of monominergic neurotransmitter systems[2] similar to those induced by chlordimeform through gestational and lactational exposure. However, chlordimeform is a very week inhibitor of MAO, but presents similar permanent regional and sexual dependent effects which suggest that MAO inhibition could not support the alterations in dopaminergic and serotoninergic systems observed in the present study, and thus in formamidine pesticides, confirming that other mechanisms are implicated.

On the other hand, it has been also described that steroids play a role in the development of catecholamine systems[21-24], and may play a critical role in mammalian brain developmental of both sexes[25]. In this sense chlordimeform has been reported to disruptor different steroids hormones[8], which could also contribute to the permanent effects observed. Moreover, chlordimeform could also affect the neuronal cell replication, differentiation, synaptogenesis and axonogenesis, steroid metabolism and functional development of neurotransmitter systems, effects that could result in behavioral alterations observed in previous studies after developmental exposure to chlordimeform[26]. The loss of dopaminergic and noradrenergic projections could also play an important

role in the behavioral alterations. Prenatal exposure to chlordimeform may result in either direct damage or enhanced vulnerability of the neurotransmitter systems to future toxic insult.

Given that the dopamine and noradrenaline systems alterations in the brain regions of our study, as well as the serotonin system alteration from our previous study[2] with chlordimeform (frontal cortex, striatum, and hippocampus) was the same as those affected by amitraz[1] it can be inferred that the mechanism by which formamidines alter CNS development is similar. Moreover, these brain regions participate in the regulation of learning and memory processes[27-32], thus, it could be considered that these processes could be compromised by exposure during gestation and lactation to formamidines. In addition, the dysfunction in serotonin and dopamine systems are involved in appetite, affective, neuropsychiatric disorders[33-36], among others, which could be also induced by formamidine exposure during development. Further studies are needed to test these functions to confirm that alteration of these neurotransmitter systems is the cause of some of these dysfunctions.

Conclusion

The results of present work show that formamidines, particularly chlordimeform, cause developmental neurotoxicity at monoaminergic neurotransmitter systems level and confirm that other mechanism aside from MAO inhibition is implicated. Further studies are required to determine the possible mechanisms through which formamidines induced these effects, specifically the hormonal disruption effects. a pathologic examination in the affected regions to determine the effect on the number of neurons is also needed, to determine if there is a reduction in innervation. Prenatal exposure to formamidine may result in either direct damage to or enhanced vulnerability of the neurotransmitter systems to future toxic insult. Due to the fact that monoaminergic neurotransmitters dysfunctions are related with appetite, affective, neurological and psychiatric disorders, behavioral studies of formamidines are also needed to clarify the outcomes of long-term alterations in these monoaminergic neurotransmitters systems. Currently, new molecules with therapeutic application are being developed as N-hydroxy-N-(4-butyl-2-methylphenyl) formamidine (HET0016) with protective effects against cardiovascular and cerebrovascular diseases. Until now the risk assessment of the family of these compounds has been taken from the standpoint of carcinogenesis. In view of these results and our previous results it might be appropriate to reconsider the risk assessment of the members of this family based not only on their possible carcinogenic effects but also in the neurotoxic effects during development. The results reported in this study are of great importance and should be incorporated into the risk assessment of pesticides formamidines group.

Compliance with ethical standards

All experiments were performed in accordance with European Union guidelines (2003/65/CE) and Spanish regulations (BOE 67/8509-12, 1988) regarding the use of laboratory animals.

Conflict of interest

The authors declare that there are no conflicts of interest.

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