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ORIGINAL

Striatal alteration of monoaminergic neurotransmitters systems in rats, after prenatal and postnatal exposure to chlordimeform, through testosterone and estradiol disruption

Alteración de los sistemas de neurotransmisores monoaminérgicos en el cuerpo estriado de rata tras exposición pre y posnatal a clordimeformo por disrupción de las hormonas estradiol y testosterona

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Abstract

Introduction: Chlordimeform, as well as other formamidine pesticides, induce permanent sex- and region-dependent effects on development of monoaminergic neurotransmitter systems. These effects could be related to monoamine oxidase (MAO) inhibition. However, chlordimeform is a very weak MAO inhibitor, which suggest that other mechanism should be involved. In this regard, chlordimeform alters testosterone and estradiol levels in frontal cortex, which lead to a disruption of the enzymes' expression that mediate the synthesis and metabolism of monoaminergic neurotransmitters systems. Therefore, an alteration of these hormones and enzymes in the other brain regions altered could also mediate the effects observed.

Objectives and methods: In order to confirm that the formamidines produce permanent alterations of the monoamine neurotransmitter systems, through disruption of sex hormones in the striatum, by alteration of the expression of the enzymes that synthesize and/or metabolize these neurotransmitters, we evaluated, in striatum of male and female rats, the effect on the levels of testosterone and estradiol at 11 days of age, as well as the expression of MAO, COMT, BDH, TH, TRH, and AD enzymes at 60 days of age after maternal exposure to chlordimeform (5 mg / kg body weight).

Results: Chlordimeform induced a significant decrease in testosterone and estradiol levels in striatum of rats at 11 days of age. We observed sex interaction with treatment in the content of T and E2. We determined a bigger increase in the expression of BDH [44,65% (P<0,001)] enzyme in females than in males. Chlordimeform treatment did not alter the expression of MAO and AD enzymes, but decreased the expression of the enzymes COMT, BDH, TH and TRH in both males and females.

Conclusions: The present findings indicate that after maternal exposure to formamidines, in general, and chlordimeform, in particular, a permanent alteration of monoaminergic neurotransmitters, through alteration of the enzymes that synthetize these neurotransmitters, mediated by sex hormones disruption, in striatum, is induced.

Keywords: Chlordimeform; formamidines; neurodevelopmental toxicity; COMT; BDH; TH; TRH; rats; human risk assessment

Resumen

Introducción: El clordimeformo, así como otros plaguicidas formamidínicos, induce alteraciones permanentes de los sistemas de neurotransmisores monoaminérgicos de forma región y sexo dependiente. La inhibición de la monoamino oxidasa (MAO) por parte de estos compuestos, podría mediar estos efectos. Sin embargo, el clordimeformo es un inhibidor muy débil de la MAO lo que sugiere que otro mecanismo debería estar implicado. En este sentido, se ha descrito que el clordimeformo altero los niveles de las hormonas testosterona y estradiol en la corteza frontal, lo que condujo a la alteración de la expresión de las enzimas que sintetizan y metabolizan estos neurotransmisores. Por lo tanto, una alteración de estas hormonas y enzimas en las otras regiones afectadas podría también mediar los efectos observados en las mismas.

Objetivos y métodos: Con el objetivo de confirmar que las formamidinas produce alteraciones permanentes de los neurotransmisores monoaminérgicos, a través de la disrupción de las hormonas sexuales a nivel del cuerpo estriado por alteración de la expresión de las enzimas que sintetizan y/o metabolizan estos neurotransmisores, se evaluaron los efectos, en el cuerpo estriado de ratas macho y hembra, sobre los niveles de testosterona y estradiol a los 11 días de edad, así como sobre la expresión de las enzimas MAO, COMT, BDH, TH , TRH, y AD a los 60 días de edad tras la exposición maternal al clordimeformo (5 mg/kg de peso corporal).

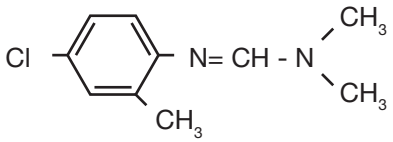
Resultados: El clordimeformo indujo una disminución significativa de los niveles de testosterona y estradiol en el cuerpo estriado de las ratas descendientes a la edad de 11 días. Se observó una interacción por sexo con el tratamiento en el contenido de T y E2. Además se observó una mayor expresión de la enzima BDH [44,65% (P<0,001)] en las hembras que en los machos. El tratamiento con clodimeformo no alteró la expresión de las enzimas MAO y AD, pero indujo una disminución en la expresión de las enzimas COMT, BDH, TH y TRH tanto en machos como en hembras.
Conclusiones: Los presentes resultados indican que las formamidinas, en general, y el clordimeformo, en particular, inducen, tras la exposición maternal, una alteración permanente de los sistemas de neurotransmisores monoaminergicos en el cuerpo estriado, a través de la alteración de las enzimas que metabolizan y sintetizan estos neurotransmisores, mediada por la alteración de las hormonas sexuales.

Palabras clave: Clordimeformo; formamidinas; neurotoxicidad en el desarrollo; testosterona; estradiol; COMT; BDH; TH; TRH; ratas; evaluación del riesgo para el hombre.

Introduction

Formamidine pesticides as amitraz have been described to produce permanent alterations on the development of central nervous system (CNS) such as those that affect monoamine neurotransmitter systems[1]. Chlordimeform [N2-(4-chloro-o-tolyl)-N1.N1-dimethylformamidine] (**Figure 1**), another member of formamidine pesticides family, has also been reported to induce permanent alterations of serotoninergic, noradrenergic and dopaminergic systems in a region- and a sex-dependent way[2,3].

Figure 1: Chlodimeform chemical structure (C10H13Cl N2).



The mechanisms by which these effects occur are not completely understood. Monoaminergic neurotransmitters play a role during development, defined as "morphogenetic"[4-7]. Changes in catecholamines levels during brain development may induce both structural and functional alterations[8]. Formamidines have been reported to inhibit monoamine oxidase (MAO)[9-10], which participates in metabolic inactivation of the neurotransmitters serotonin (5-HT), norepinephrine (NE), and dopamine (DA). Therefore, this action could mediate the effects observed on monoaminergic neurotransmitters. However, chlordimeform is a very weak MAO inhibitor, which suggest other mechanisms are involved in these effects.

Otherwise, formamidines are endocrine disruptors and particularly, chlordimeform and amitraz have been reported to alter serum hormone levels[19, 20]. Changes in NE, DA and 5-HT and its metabolites levels observed in rats' brain after formamidines exposure could be attributed to a possible effect on sex steroid hormones that modulate the expression of enzymes such as tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH), tryptophan hydroxylase (TRH), MAO, catechol-O-metyltransferase (COMT), aldehyde dehydrogenase (AD), aldehyde reductase (AR) required for synthesis and metabolism of these neurotransmitters[11-18]. In this regard, after prenatal and postnatal exposure to chlordimeform, a disruption in testosterone and estradiol levels in frontal cortex was observed, which lead to changes in the expression of TH and TRH[21]. Thus, these mechanisms could explain chlordimeform's, as well as other formamidine pesticides', effects observed on monoaminergic neurotransmitters systems.

Therefore, we performed a study to establish if maternal exposure to chlordimeform during gestation and lactation induces permanent alterations on the enzymes that synthetize and metabolize 5-HT, NE and DA neurotransmitters in striatum, at adult age, through sex hormones disruption, which could explain the effect observed on these neurotransmitters in a sex- and region-dependent way. Chlordimeform was chosen because it is the most representative compound of formamimidines family, 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 to MAO inhibition.

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. Eight pregnant Wistar rats were housed individually in polycarbonate cages and were assigned randomly to two experimental groups: a chlordimeform treatment group (n = 4) and a control group (n = 4).

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 pre-

liminary study that indicated that this dose was the higher one that did not cause weight loss or mortality, reduction of food or water intake as well as did not induce haematological modifications or other clinical histopathological signs of overt toxicity. 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 behaviour. 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 twenty-four pups, twelve males and twelve 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 PN11, for the analysis of brain's testosterone and estradiol levels and at PN 60, for the analysis of MAO A, MAO B, COMT, BDH, AD, TH and TRH gene expression, 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 striatum was a rapidly dissected out at 4ºC[22], since this brain region was previously describe to present sex differences in the effect observed on these neurotransmitters systems and to be one of the most affected[2,3]. Tissues were rapidly weighed and stored at -80ºC until analysis. All data were collected by experimenters blind to the treatment condition of the offspring.

Estradiol and testosterone quantification

Estradiol and testosterone content were measured in striatum from treated animals in order to determine whether sex hormones are altered by chlordimeform exposure. Estradiol and testosterone content in the striatum was measured using an enzyme immunoassay kit (Estradiol EIA Kit, Cayman Chemical Company, MI, USA), according to the manufacturer's instruction. Tissues were homogenized in 300 µl of an equal mixture of ethyl acetate and 0.1 M phosphate-buffered saline. The homogenates were centrifuged at 21,000 g for 15 min at 4ºC. The resulting mixture was then incubated in a MeOH/dry ice bath to solidify the aqueous phase (bottom) and the organic phase was eluted into a new tube. The ethyl acetate portion was collected and dried. The dried material was reconstituted in 120 µl EIA buffer, and 100 µl of the sample was used for EIA at duplicate. ELISA values were obtained (pg/ml) and corrected for weigh tissue (mg/ml), producing a final unit of pg/mg and presented as a percentage of the untreated control.

Real-time PCR analysis

The MAOA, MAOB, COMT, AD, TH, TRH and DBH expression was measured in striatum tissue from control and chlordimeform treated animals in order to determine whether chlordimeform, through sex hormones disruption, alters permanently the expression of these enzymes. Total RNA was extracted using the Trizol Reagent method (Invitrogen, Madrid, Spain). The final RNA concentration was determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Madrid, Spain), and the quality of total RNA samples was assessed using an Experion LabChip (Bio-Rad, Madrid, Spain) gel. First-strand cDNA was synthesized with 1000 ng of cRNA by using a PCR array first strand-synthesis kit (C-02; SuperArray Bioscience, Madrid, Spain) in accordance with the manufacturer's instructions and including a genomic DNA elimination step and external RNA controls. After reverse transcription, QPCR was carried out using prevalidated primer sets (SuperArray Bioscience) for mRNAs encoding MAOA (PPR46359A), COMT (PPR06789A), AD (PPPR43520B), TH (PPR45220F), TRH (PPR48244A), DBH (PPR52652A), and ACTB (PPM02945B). ACTB was used as an internal control for normalization. Reactions were run on a CFX96 using Real-Time SYBR Green PCR master mix PA-012 (SuperArray Bioscience). The thermocycler parameters were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 72°C for 30 seconds. Relative changes in gene expression were calculated using the Ct (cycle threshold) method. The expression data are presented as actual change multiples[23].

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

Estradiol and testosterone quantification

Oral treatment with chlordimeform to dams during the gestation period from day 6 to day 21 and during lactation from day 1 to day 10 affected the content of T and E2 in the striatum region of offspring rats at the age of 11 days. The content of T (ng/g tissue) in the region of striatum in the control group and treated group is presented in **table I**.

Table I: Tissue T (pg/ml) content determined in striatum from male and female rats at 11 days of age treated with vehicle or chlordimeform (5 mg/kg bw orally on days 6 to 21 of pregnancy and 1 to 10 of lactation).

Frontal Cortex				
Animal	**Control group Males**	**Treated group Males**	**Control group Females**	**Treated group Females**
1	201,87	164,87	213,69	152,84
2	210,55	175,98	206,77	143,98
3	209,70	172,79	209,31	156,42
4	213,73	178,56	201,71	147,33
5	205,96	169,45	216,76	149,87
6	215,91	173,28	206,68	146,87
Mean ± SEM	**209,62±1,88*** **	**172,49±1,97*** ** **(-17,71%)**	**209,15±2,20*** **	**149,55±1,84*** ** **(-28,50%)**

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*<0.001 levels compared with the control group.

The content of E2 (ng/g tissue) in the region of the striatum of the control group and the treated group is presented in **table II**. The results expressed in **tables I** and **II** show that in 11 days old rats treated during gestation days 6-21 and during lactation days 1-10 through their mothers, a statistically significant loss of E2 and T content in the striatum compared to control animals was produced. A sex interaction with treatment in the content of T and E2 was observed (**Figure 2**). In striatum, the loss observed of E2 content was 48,01% (P<0,001) and 58,95% (P<0,001) in males and females, respectively, and the loss in the content of T was 17,71% (P<0,001) and 28,50% (P<0,001) in males and females, respectively (**Figure 2**).

Real-time PCR analysis

Oral treatment with chlordimeform to dams during the gestation period from day 6 to day 21 and during lactation from day 1 to day 10 affected the COMT, BDH, TH and TRH gene expression of offspring rats at the age of 60 days. In 60 days old rats treated during gestation days 6-21 and during lactation days 1 to 10 a decrease in the expression of COMT [23,35% (P<0,01)], BDH [26,15% (P<0,01) and 60,64% (P<0,001) in males and females, respectively], TH [29,46% (P<0,01)] and TRH [43,24% (P<0,01)] enzymes in striatum with respect to control animals was observed. No effect on gene expression of MAO, and AD enzymes was observed (**Figure 3**). A sex difference in BDH gene expression was observed, being higher the expression of BDH [44,65% (P<0,001)] enzyme in female than in male rats (**Figure 4**).

Discussion

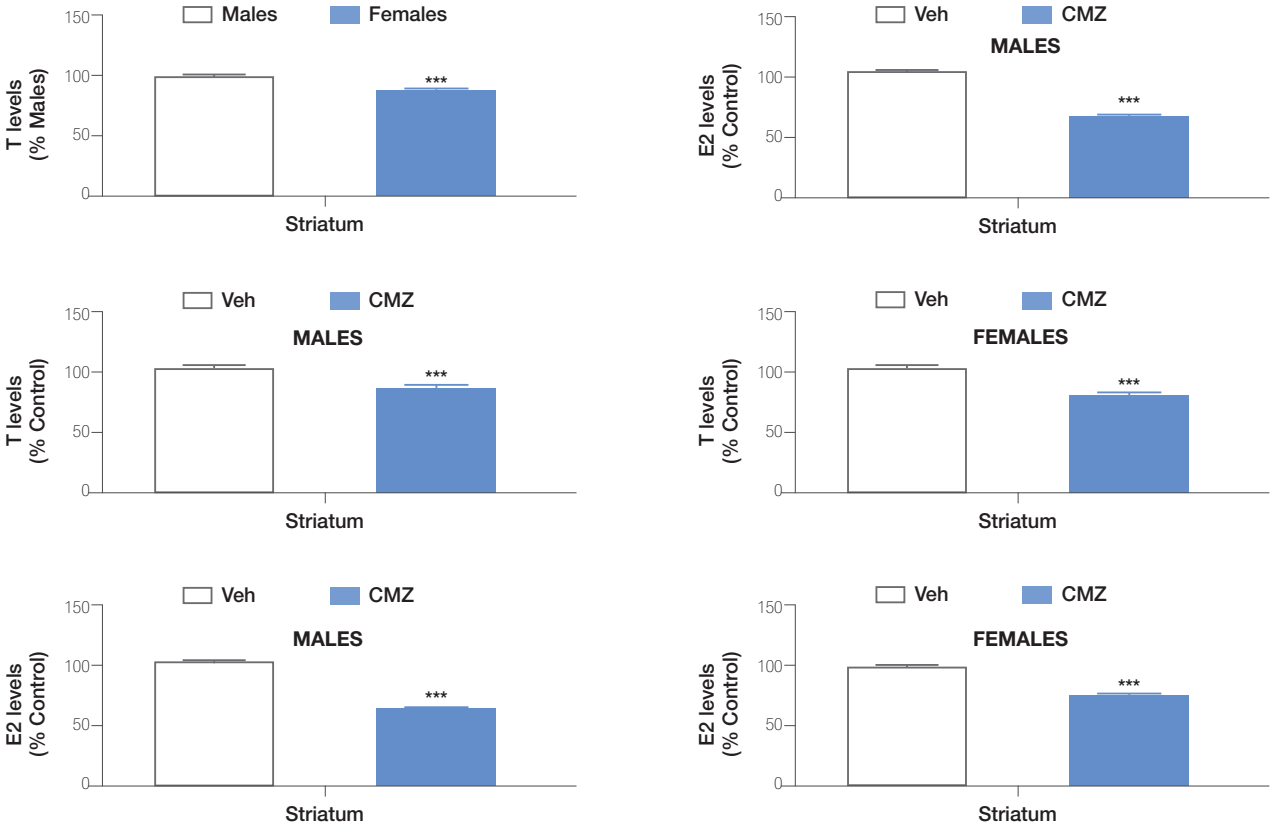
Chemical exposure of dams during pregnancy or lactation could induce developmental neurotoxic effects that include alterations in behaviour, neurohistology, neurochemistry and/or gross dysmorphology of CNS, which are manifest in the adulthood. Previous studies described that formamidines induce permanent alteration in developing monoamine neurotransmitter systems in a sex- and region-dependent way[1-3]. Specifically, chlordimeform has been reported to induce an alteration of 5-HT, DA and NE neurotransmitters and their metabolites in a sex-dependent way in only the regions of frontal cortex, striatum and hippocampus[2,3]. The mechanism by which these permanent effects on monoaminergic systems take place is not completely understood, but monoamine neurotransmitters

Table II: Tissue E2 (pg/ml) content determined in striatum from male and female rats at 11 days of age treated with vehicle or chlordimeform (5 mg/kg bw orally on days 6 to 21 of pregnancy and 1 to 10 of lactation).

Frontal Cortex				
Animal	**Control group Males**	**Treated group Males**	**Control group Females**	**Treated group Females**
1	67,84	36,87	60,76	21,87
2	64,88	38,33	68,94	24,81
3	59,98	29,76	59,64	27,98
4	63,85	35,91	68,64	30,34
5	68,98	31,94	63,81	26,95
6	73,29	34,55	62,76	25,93
Mean ± SEM	**66,47±1,88*** **	**34,56±1,30*** ** **(-48,01%)**	**64,09±1,60*** **	**26,31±1,18*** ** **(-58,95%)**

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*<0.001 levels compared with the control group.

Figure 2: Tissue T and E2 (pg/ml) content determined in striatum from male and female rats at 11 days of age treated with vehicle or chlordimeform (5 mg/kg bw orally on days 6 to 21 of pregnancy and 1 to 10 of lactation).

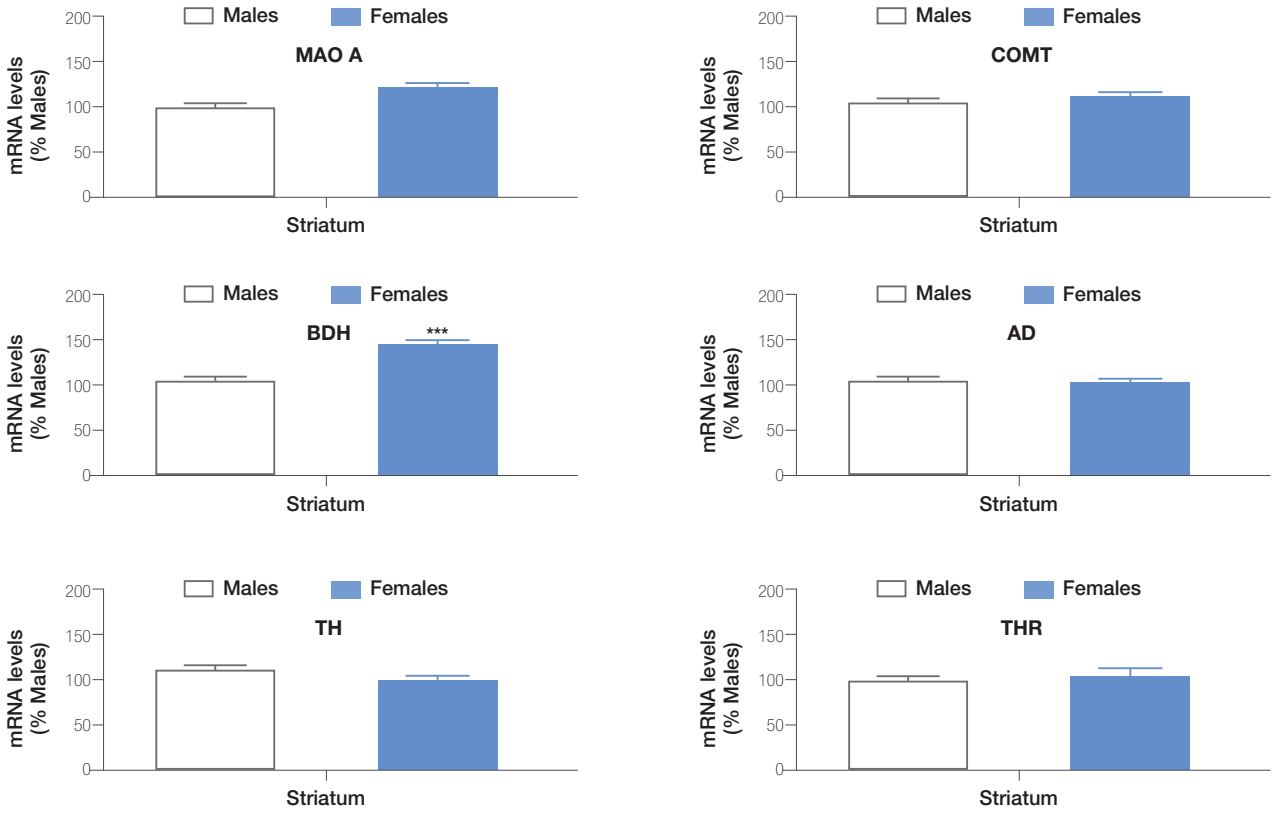


regulate brain development prior to assuming their roles as transmitters in the mature brain[24-26], thus any circumstance that affects these neurotransmitters in the developing brain can alter the final structure and function of that brain. Since the endogenous levels of 5-HT, DA and NE are highly regulated 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 frontal cortex[27], but not in the dopaminergic and noradrenergic innervation, which suggests that besides MAO inhibition other mechanism should be implicated in the alteration observed. However, chlordimeform is a very weak MAO inhibitor[28-30], but presents similar permanent regional and sexual dependent effects than amitraz, which is a potent MAO inhibitor[9]. These data suggest that MAO inhibition could not produce the alterations in monoaminergic neurotransmitters systems observed, confirming that other mechanisms are involved.

Otherwise, steroids play a role in the development of catecholamine systems[31-34], and play a critical role in mammalian brain developmental of both genders[35]. 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 a decrease in T and E2 levels at PN11, which is the critical period of time when sexual differentiation takes place, in male and female rats' brain. This treatment produced also a permanent reduction of the COMT, BDH, TH and TRH gene expression, which catalyse the synthesis and metabolism of monoaminergic neurotransmitters, at 60 days of age in male and female rats' striatum. Previously, chlordimeform has been reported to decrease T and E2 levels at PN11 and a reduction of the TH and TRH gene expression at PN60 in frontal cortex[20], which support the effect observed. The sex and region differences in the enzymes altered in this region correspond with the alteration observed on the monoaminergic neurotransmitters in this region, which could explain the effect observed.

Sex hormones' effect on monoaminergic and indolaminergic neurotransmitters in CNS includes synthesis, vesicular and/or synaptic release and metabolism regulation[36].The sex hormones provenance in the brain, could be from gonads or from endogenous synthesis, as previously described, whose contribution to the final effect depends on the region and sex steroid hormone[37-40]. Estradiol alters the levels of enzymes that synthesize DA, NE and 5HT, as well as those that degrade these

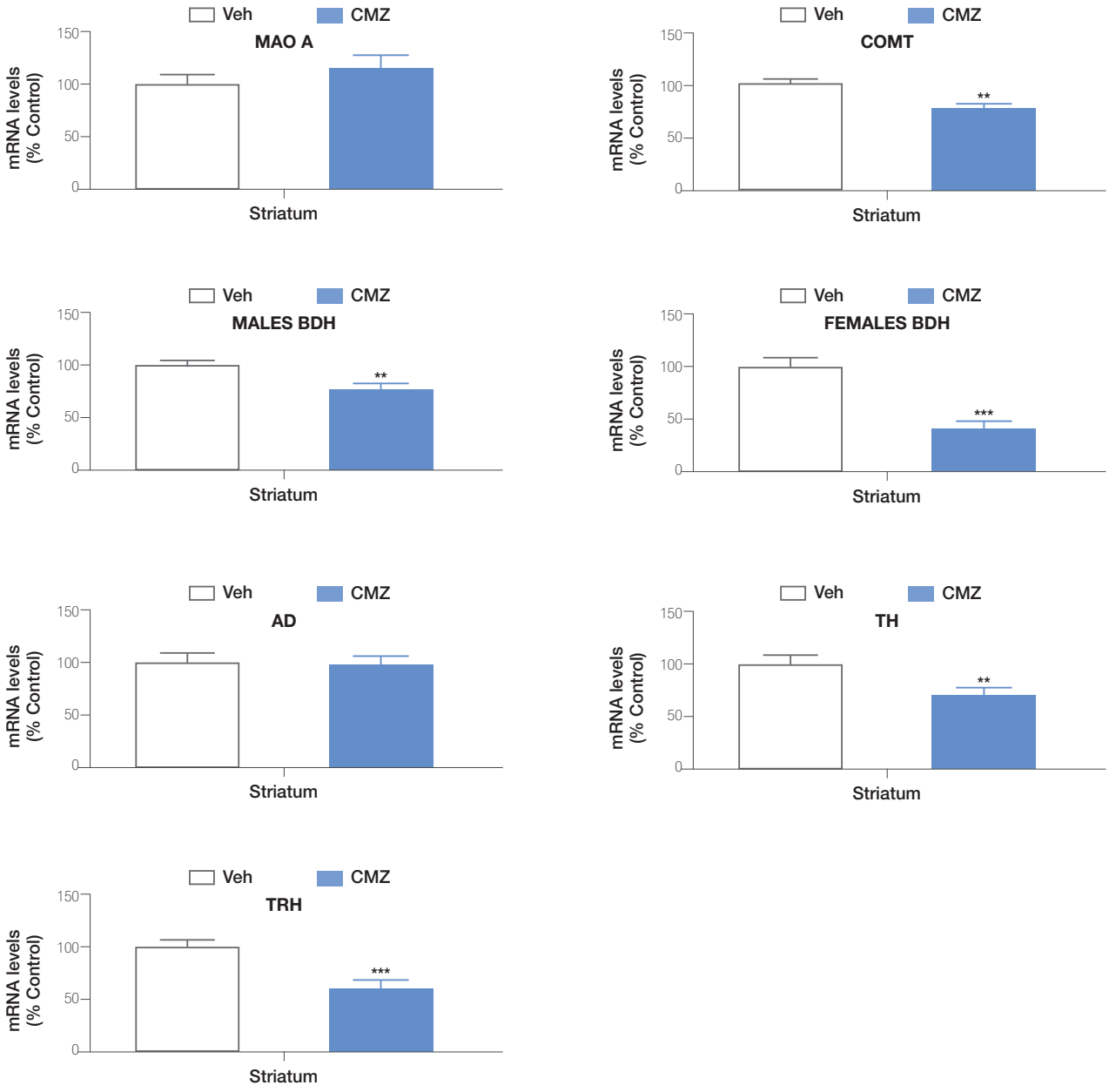
Figure 3: Sex difference results from real-time PCR targeting MAO, COMT, BDH, AD, TH y TRH genes after chlordimeform treatment in male and female rats. MAO, COMT, BDH, AD, TH y TRH gene expression was compared to male rats results. Each bar represents mean ± SD of 6 samples. Levels were measured using QPCR. ACTB was used as an internal control. ***p ≤ 0.001,**p ≤ 0.01, significantly different from males.



neurotransmitters[12,14,16,41-42]. E2 elevated mRNA levels of TH, the first and major rate limiting enzyme in catecholamine biosynthesis[42] and enhanced TRH mRNA expression[12]. In addition, T and DHT regulated the synthesis and metabolism of monoamines[17]. In this sense, T and DHT increased TH protein and COMT, MAO-A and MAO-B mRNAs[15]. In the same way, DHT decreased neurotransmitter turnover of DOPAC/DA, MHPG/NE, and 5-HIAA/5-HT of gonadectomized animals[13]. These previous data support that the disruption in sex hormones observed, mediate the effects observed on these enzymes after chlordimeform treatment, and so, on the monoaminergic neurotransmitters. However, we cannot rule out that an alteration of monoaminergic neurotransmitters transporters, which have been shown to be regulated by estradiol[43-46], might contribute to the effect observed.

Furthermore, other possible mechanisms that may contribute to the permanent alterations observed on monoaminergic neurotransmitters systems could be a direct action of chlordimeform on neuronal cell replication, differentiation, axonogenesis and synaptogenesis and functional development of neurotransmitter systems, effects that could result in behavioural alterations observed in previous studies after developmental exposure to chlordimeform[47]. The loss of dopaminergic, serotoninergic and noradrenergic projections could also play an important role in the behavioural and motor alterations. In this regard, striatum participates in the regulation of motor control and learning and memory processes, among other actions[48-51], thus, it could be considered that these processes could be compromised by exposure during gestation and lactation to formamidines. Moreover, neural functions like affect, anxiety, mood, fear and cognitive function are modulated by estradiol, predominantly enhancing learning and memory, in addition to its well-documented role in reproduction[52]. Therefore, the neurotoxic effects observed by chlordimeform or amitraz exposure such as behavioral effects as hyperreactivity to external stimuli, aggressiveness, and motor incoordination, among others[47,53], could be mediated by the alteration observed in these neurotransmitters. Neurofunctional disorders such as schizophrenia, aggressive behaviour, autism spectrum disorder and attention deficit hyperactivity disorder has been associated with imbalance in dopamine and other neurotransmitters in the developing brain[54-59], so these alterations could also lead to development of some of these neurological disorders after formamidines exposure. Further studies are needed to test whether these other mechanisms described could be involved in the effects observed and to confirm that

Figure 4: Results from real-time PCR targeting MAO, COMT, BDH, AD, TH y TRH genes after chlordimeform treatment in male and female rats. MAO, COMT, BDH, AD, TH y TRH gene expression was compared to controls. Each bar represents mean ± SD of 6 samples. Levels were measured using QPCR. ACTB was used as an internal control. ***p ≤ 0.001, **p ≤ 0.01 significantly different from controls.



alteration of these neurotransmitter systems is the cause of some of these dysfunctions.

DA, 5-HT and NE systems alterations observed after chlordimeform exposure in the frontal cortex, striatum, and hippocampus were similar between them[2,3]. Moreover, the testosterone and estradiol hormone levels disruption produced after chlordimeform exposure in the frontal cortex and striatum was also similar, as well as the effect on monoaminergic neurotransmitters regulating enzymes gene expression, except for COMT and BDH. These results suggests that the mechanisms through which monoaminergic neurotransmitters systems are altered in the brain regions affected after chlordimeform exposure is produced by the alteration in the expression of these enzymes, mediated through sex hormones disruption. In addition, the effects observed on DA, 5-HT and NE systems after amitraz exposures were also the same as those observed after chlordimeform exposure[1], suggesting that these mechanisms are the same in chlordimeform and amitraz in particular, and in formamidines in general. Further studies are needed to confirm whether this mechanism and others, probably involved in these effects, are the same in all brain regions studied and for all formamidines.

Conclusion

In summary, our results suggest that the mechanism by which the alterations in the development of the monoaminergic neurotransmitter systems in striatum is mediated through disruption of estradiol and testosterone levels, which produced a permanent alteration of the expression of some of the enzymes that synthetize and metabolize these monoaminergic neurotransmitters. Further studies are required to check whether other hormones are also involved in these effects and to determine whether they act directly on expression of the affected enzymes or through induction of other genes that can regulate their expression. Otherwise, it should be determined whether there is a reduction in innervation in the regions affected that could also contribute to the effect observed. Moreover, it should be determined if these mechanism are the same in all formamidines and in all brain regions affected by them. 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 formamidine molecules with therapeutic application are being developed. 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 mediated by endocrine disruption. The results reported in this study are of great importance because they could lead to a better understanding of the mechanisms responsible for producing the neurotoxic alterations 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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