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ULTRASONIC VOCALIZATIONS IN PUPS PRENATALLY EXPOSED TO ENVIROMENTAL FACTORS

TRABAJO FIN DE MÁSTER

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Continuará…

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Resumen

Los trastornos relacionados con el espectro autista (TEA) se caracterizan por un déficit en la interacción social y la comunicación, así como por la ejecución de comportamientos repetitivos o estereotipados. Su heterogeneidad etiológica podría ser responsable de la heterogeneidad de la sintomatología dentro del espectro. Entre los factores medioambientales que aumentan el riesgo de desarrollar TEA se ha documentado la exposición prenatal a contaminantes. Los pesticidas organofosforados en general, y el clorpirifós (CPF) en particular, han sido fuertemente relacionados con el desarrollo del autismo, o al menos con la desregulación del comportamiento social. Con el objetivo de analizar este comportamiento en crías prenatalmente expuestas a CPF, se compararán los datos con un grupo control y un modelo animal TEA validado, como es el ácido valproico (AVP). Para ello, 25 hembras fueron expuestas durante el 12.5GD, y sus crías pasaron el procedimiento de inducción de USV mediante procedimiento de separación de la madre el día PND7. Al analizar los resultados se observó un efecto del tratamiento para los grupos CPF y AVP, tanto en la latencia de la primera llamada como en el número de vocalizaciones ultrasónicas (USV), que permiten aportar evidencias de una alteración de la interacción social y la comunicación con la madre. Los resultados también indican que los efectos son sexualmente dimórficos, y que el aumento final de los USV se debe a variables no sociales.

Abstract

Autism Spectrum Disorders (ASD) are characterized by deficits in social interaction and communication as well as repetitive or stereotyped behaviors. The etiological heterogeneity could be responsible for the symptomatology heterogeneity within the ASD spectrum. Among the environmental factors that increase the risk of developing ASD, prenatal exposure to contaminants has been documented. Organophosphorus pesticides in general and chlorpyrifos (CPF) in particular have been strongly related to the development of autism, or at least to the deregulation of social behavior. In order to analyze this behavior in pups prenatally exposed to CPF, the data will be compared with a control group and a validated animal model of ASD (valproic acid) (VPA). For this, 25 females were exposed during 12.5GD, and their offspring passed the isolated from dam protocol for ultrasonic induction at PND7. A treatment effect was observed for the CPF and AVP groups, both in the latency of the first vocalization and in the number of ultrasonic vocalizations (USV). Allowing to provide evidence of a social interaction and

communication alteration. The results also indicate that the effects are sexually dimorphic, and that the final increase of the USV is due to nonsocial variables.

Keywords: ultrasonic vocalizations, chlorpyrifos, valproic acid, prenatal, autism, animal models

Abbreviations

AChE: acetylcholinesterase; ASD: Autistic Spectrum Disorder, AVP: Valproic acid, ChE: cholinesterase, CNT: Control, CPF: Chlorpyrifos, DNA: Deoxyribonucleic Acid, DSM: Diagnostic and Statistical Manual of Mental Disorders, GD: Gestational Day, OP: Organophosphate Pesticide, PND: Postnatal Day, s.c.: subcutaneous, USV: Ultrasonic Vocalization.

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1. INTRODUCTION

The Diagnostic and Statistical Manual of Mental Disorders (DSM) in its latest edition (DSM-V) (2013), characterizes autism for its persistent deficits in social interaction and communication across multiple contexts, and repetitive or restrictive patterns of behavior. These symptoms are usually followed by other comorbid ones such as epilepsy, hyperactivity, attention deficit, mental retardation, sleep problems, gastrointestinal and hypo or hyper-sensory problems, which in turn may vary the level of severity of these symptoms (Landrigan, 2010; Mabunga, Gonzales, Kim, Kim & Shin, 2015).

At present, it is easy to find numerous studies that deal with the increase in the prevalence of this set of disorders (Federación Autismo Andalucía, n.d; Baio, 2014; Villegas & Sánchez, 2014 Mabunga et al., 2015; Canals, Hernández-Martínez, Morales-Hidalgo, Roigé-Castellví & Voltas, 2018), which speak of a global increase that has multiplied by thirty since the first epidemiological studies, in Europe at that time, one in every 2,500 children (Federación Autismo Andalucía, n.d; Baio, 2014). A recent study with Spanish children shows a prevalence of 1.55% in preschool and 1% in children of school age (Canals et al., 2018). Although there are also authors who question the extent to which these data reflect a real increase in the prevalence of the spectrum and not better evaluation respect to the sixties-seventies (Federación Autismo Andalucía, n.d; Gillberg & Wing, 1999; Canals et al., 2018).

However, despite the increase in prevalence and the reformulation of the evaluation method, ASD continue to have a little-known origin. Although it is true that there is evidence that supports a polygene influence, it does not reflect the totality of the cases. Certain studies with monozygotic and dizygotic twins confirm a concordance of 66%, and up to 70% in monozygotic twins, leaving a margin for environmental factors (Folstein & Rosen-Sheidley, 2001, Landrigan, 2010, De Felice, Scattoni, Ricceri, & Calamandrei, 2015; Mabunga et al., 2015). Among those environmental factors that increase the risk of developing ASD, viral infections during pregnancy, preterm birth or prenatal exposure to contaminants have been documented (De Felice et al., 2015).

For approximately one decade, the number of data that support the evidence of the neurotoxicity of organophosphate pesticides (OP) has been increasing, focusing with special attention on chlorpyrifos (CPF), for being one of the most widely used in the world

(Levin, Addy, Nakajima, Christopher, Seidler & Slotkin, 2001, Venerosi, Ricceri, Scattoni & Calamandrei, 2009, Rauh et al., 2012, Venerosi et al., 2015). This is a non-persistent pesticide (Venerosi et al., 2009) with a half-life of 3 days (Eaton et al., 2008), whose toxicity was documented after acute poisoning in adults (Venerosi et al., 2009) by the "cholinergic syndrome". Cholinergic syndrome consists of increased sweating and salivation, bronchoconstriction, miosis, increased gastrointestinal motility, diarrhea, tremors, muscle spasms, as well as various effects on the central nervous system (Holmstedt, 1959; Lotti, 2010; Costa, 2018). There have even been reported cases of death as a result of respiratory failure (Costa, 2018). Because of this, many of the non-agricultural uses have been eliminated in the USA in 2001 and the UE in 2005. However, human direct and indirect exposure occur. The primary route of exposure is via food ingestion that contains CPF residues, but in agricultural communities, exposure pathways also include dermal contact and inhalation (Burns, McIntosh, Mink, Jurek & Li, 2013; Lee, Eriksson, Fredriksson, Buratovic & Viberg, 2015; Venerosi et al., 2015; Lan, Kalimian, Amram & Kofman, 2017).

Human and animal studies have shown that young children and animals are more sensitive than adults to the cholinergic toxicity of CPF, this could be explained by its immaturity, formation and development state (Eaton et al, 2008, Venerosi et al., 2015; Lan et al., 2017). Several epidemiological studies in the literature that have found effects in the neurobiological maturation of children after prolonged exposure to CPF (Venerosi, Calamandrei & Ricceri, 2006; Rosas & Eskenazi, 2008; Lan, Stein, Portillo, Toiber & Kofman, 2019), among these effects are the reduction in infant body length and weight, delay in cognitive and psychomotor development, perseverative developmental disorders, and attention, working memory and social skills deficits (Rauh et al., 2006; Lan et al., 2017). Other studies speak of a significant association between prenatal exposure and structural changes in the human brain (Rauh et al., 2012), as well as a positive relationship between ASD and prenatal proximity to CPF application during the second and third trimester of gestation (Venerosi, et al., 2006; Shelton et al. al., 2014; Venerosi et al., 2015; Lan et al., 2019).

Besides, data from laboratory animals showed that even subtoxic exposure to CPF, despite protecting the offspring against the inhibition of cholinesterase (ChE), continues to affect the early development of the nervous system. At subtoxic doses CPF caused inhibition of DNA and protein synthesis, decreased cell density in brain,

cerebellum and brainstem of neonatal rats, as well as affected expression levels of critical genes involved in fetal brain development, including genes involved in motor abilities, learning, neuronal communication, growth and plasticity, suggesting that CPF has non-cholinergic mechanisms of action, as well (Eaton et al, 2008; Lan et al., 2017). Perinatal exposure to CPF also affects levels of neuropeptide-hypothalamic oxytocin and vasopressin. Hypothalamic neuropeptides act as key regulators of anxiety, aggression, as well as various aspects of social behavior in mammals. All this makes pregnant women a potentially susceptible subpopulation (Eaton et al, 2008; Venerosi et al., 2015).

Unfortunately, not all the signs and symptoms of disorder are evident at the beginning of development, but are noticed when the child cannot meet certain social and/or educational demands, such as communication and social deficits or behavior patterns in ASD (Developmental Disabilities Monitoring Network Surveillance Year 2010 Principal Investigators & CDC, 2014). This fact makes especially important to find a method through which to evaluate social behavior and communication in the early stages, as an early predictor of the disease. Since animals do not have an organized language system like ours, finding a way to evaluate communication has been a challenge. Interestingly, rodents are widely used in the laboratory due to their high levels of social interaction and exploration (Kas et al., 2014, Mabunga et al., 2015), so that researchers could identify how rodents emit certain ultrasonic vocalizations (USV), above the human hearing threshold, when they tried to communicate. The frequencies of these calls vary according to age, emotional state and environmental factors (Portfors, 2007, Venerosi et al., 2009, Mabunga et al., 2015). Thus, USV have become both one of the few early markers of neurobehavioral development, and a way to evaluate early emotional responses in life. For this reason, the use of USV in the evaluation of sociability in rodents and other animals is very frequent, as well as the assessment of establishment of the dam-offspring bond (Venerosi et al., 2009; De Felice et al., 2015; Mabunga et al., 2015).

Certain studies in which USV were analyzed have found evidence that CPF significantly reduces the stress responsible for the separation of the mother and motor skills, in addition to alterations in characteristics similar to infant crying (duration of the call, peaks of frequency and latency of the first call), supporting the idea of being a marker for preclinical studies and a potential predictor of the long-term emotional and social effects of CPF also reported in adulthood (Venerosi et al., 2009).

In order to compare the results of this OP in one of the central handicaps of the spectrum, sociability and communication, we will use an already validated epigenetic animal model. Currently, one of the most widely used models to study autism under conditions of environmental risk factor in perinatal stages is valproic acid or valproate (AVP) (Belzung et al., 2005; De Felice et al., 2015; Mabunga et al., 2015). AVP is a drug for epilepsy and mood swings, with which must be extremely careful especially during critical periods such as pregnancies, since children with fetal valproate syndrome have been found to show social deficit and stereotyping (Mabunga et al., 2015). Also, Schneider et al. (2008) observed repetitive behaviors and decreased social behavior, among other long-term effects, in rats exposed to VPA on day 12.5 GD.

Given the data provided, this research group aims to compare the effects of prenatal exposure to CPF on social behavior and communication with a control group and an animal model of the autism spectrum in force to date (AVP), through an ultrasound analysis by the isolation from the dam protocol; Allowing us to delimit the superior and inferior thresholds of this behavior and to situate the effects of the CPF in absence of inhibition of the acetylcholinesterase (AChE). We expected that the behavioral repertoire of the CPF group is closer to the VPA group than the control group, this is a longer first vocalization latency and a lower USV rate than the control group, a fact that would confirm it as a possible risk factor of ASD.

2. METHODS

2.1. **Animals**

Twenty-five pregnant wistar rats (Janvier, France) and eighty-seven pups of their offspring were used for this investigation. There were three experimental groups through the experiment, first with dams: control group (n=8), CPF group (n=8) and VPA group (n=9). And then, with pups in a gender distribution 1:1: control group (n=27), CPF group (n=28) and VPA group (n=32). Animals were housed four per cage (50×15×25 cm) under a light/dark cycle of 12 hours (light on 7:00h to 19:00h) in a temperature-controlled room at 24ºC. Food (PanLab Chow) and water were available ad libitum. All testing was performed between 9:00 and 16:00 h.

The present study is part of the project PSI2017-86847-C2-1-R and was conducted in accordance with the Spanish Royal Decree 53/2013 and the European Community

Directive (2010/63/EU) for animal research and approved by the University of Almeria Animal Research Committee.

2.2. **Drugs**

Chlorpyrifos (purity>99.5%) and valproic acid (purity>98%) were purchased from Sigma–Aldrich Quimica (Madrid, Spain). CPF was prepared in dimethyl sulfoxide (DMSO) and administered at a dose of 1 mg/kg body weight, and VPA was prepared in saline and administered at a dose of 400 mg/kg body weight, both were administered subcutaneously (s.c).

The doses were selected based on literature and data nonpublished from our group because they do not induce AChE inhibition or acute toxicity (Qiao, Seidler, Padilla & Slotkin, 2002; Cezar et al., 2018).

2.3. **Apparatus**

We conducted the test in a plywood sound attenuator box, upholstered inside with a polystyrene panel and another black soundproofing foam (80x60x70 cm). An ultrasonic microphone (Dodotronic Ultramic 250K), chosen for its use in a similar investigation (Blazevic, Merkler, Persic & Hranilovic, 2017) and even used with other species (Brizio & Buzzetti, 2014), was placed inside the box about 15-20 cm above the ground. The recordings were made with the SeaWave software version 2.0 (CIMBRA) with a sampling frequency of 250 kHz, in a 16-bit format.

2.4. **Experimental procedures**

The experiment started during the pregnancy of dams, where they were divided in three groups and injected. The rest of the experiments were carried out on the offspring.

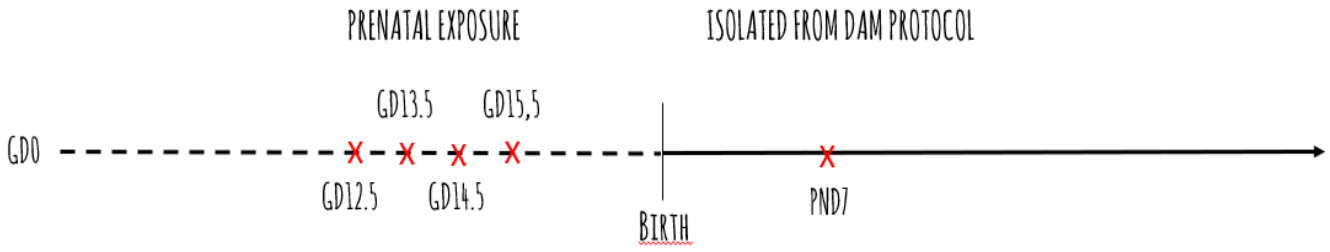

FIGURE 1: EXPERIMENTAL DESIGN. The procedures here described are marked with red x's: during the gestational period (days 12.5, 13.5, 14.5 and 15.5) the animals were exposed to different substances and after birth, on PND7 they were administered an isolation protocol to elicit the USV emission.

2.4.1. Prenatal exposure and toxicity evaluation

Animals were prenatally exposed to different substances, for this a total of twenty-five pregnant wistar rats were available, which at gestational day 12.5 (GD12.5) were divided into three groups and received a subcutaneous (s.c.) injection with the corresponding substance: vehicle (1 mg/kg) for control group, chlorpyrifos (1 mg/kg) for CPF group and valproic acid (400 mg/kg) for VPA group. The injections were repeated on days 13.5GD, 14.5GD and 15.5GD for control and CPF groups. For the VPA group, the subsequent injections were of saline because only an exposure to VPA is necessary to produce the effects.

To assess possible signs of intoxication, pups were weighed daily from postnatal day (PND10). The intakes from day 10th to 20th were used to evaluate this toxicity. Earlier weights couldn't be gotten because of mothers reaction.

2.4.2. Isolated from dam protocol (USV)

The day of birth was considered PND0 and the offspring were sexed. On PND 7 to favor the production of ultrasonic vocalizations we applied the isolated test basic protocol of Brunelli, Hofer and Shair (2002) with subtle modifications. In order to work without the aggressiveness of the mother, a polycarbonate box with similar characteristics to the one they normally housed (commented above) was prepared and the pups were introduced. This box had a thermal blanket covered with cotton from the nest with the smell of the mother, as well as sawdust and some poops extracted from the original home-cage to prevent them from noticing the lack of the mother. Then, each pup was carefully introduced into a test box and sound was recorded for 3 minutes. At the end of each recording session, the box was disinfected.

At the time of the analysis of the recordings, they did not receive any treatment, because they were analyzed directly in the software with which they were obtained (SeaWave 2.0). The recordings were divided by minutes and the number of vocalizations in each of them was counted to know the evolution of USV over time. The time it took for each rat to emit the first call in seconds or call latency and the total number of USV during the three minutes of the recording were also measured. The vocalizations were manually recorded after previous training.

A pilot test was successfully carried out with pups with which they were not subsequently experimented in order not to contaminate the results. The temperature intakes of the offspring suggested by the protocol were omitted, given that the protocol remained constant at around 25ºC throughout the test, thanks to the blanket and cotton. Also, the weighing and locomotion tests were omitted, because the weight was collected daily outside this protocol and on PND10 they passed the locomotion test *per se*.

2.5. **Data analysis**

First vocalization latency and the total number of USV during the first, the second and the third minute were analyzed with a two factors ANOVA according to Sex (male or female) and Treatment (control, CPF or AVP). Weight evolution was analyzed using repeated-measures analysis of variance (ANOVA), also according to Sex and Treatment with the day as within-subject factor respectively. Post hoc were made using the Bonferroni test, taking a level of significance for all tests of $p<0.05$.

For data analysis were used IBM SPSS Statistics version 24 and GRAPHPAD Prism version 6.0 for the graphs.

3. RESULTS

3.1. **Weight evolution**

For all treatments, the weight evolution has been progressive and significant for each day in which measurements were taken respect to the previous day ($F (4.940, 200.071) =3.555$, $p=0.004$).

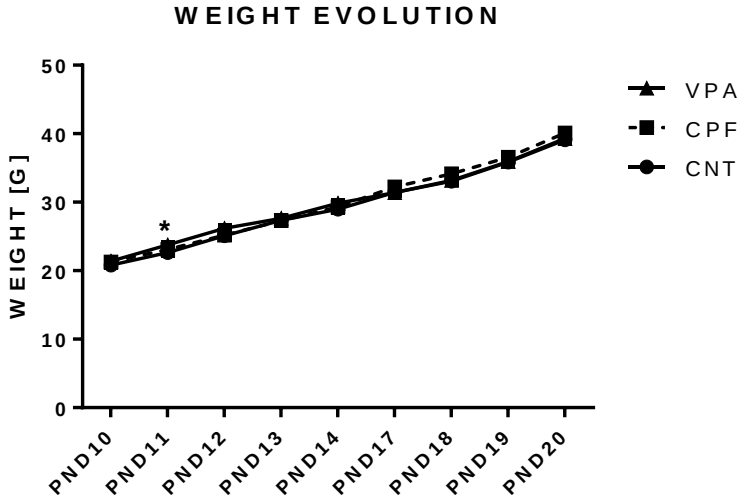

FIGURE 2: GRAPH OF WEIGHT EVOLUTION PER TREATMENT. The weight evolution for all treatments was progressive, with only a significant difference between treatments on PND11 (0.004<0.05).

The PND15 and PND16 have not been included because weights were not taken during the weekend. Differences are only found in PND11 ($F(15, 104) = 3.555$, $p=0.004$), where the control group (22.6 ± 0.03) showed weights significantly lower than the VPA group (23.8 ± 0.31) ($p=0.007$).

3.2. **USV recording**

Once the data was obtained, the extreme cases were calculated for each variable using the SPSS Statistics from IBM explore option and they were eliminated to prevent these values from seriously altering the study, masking the effects of the treatment, insomuch as we were looking to characterize the populations.

There are significant differences in first call latency into Sex ($F(2, 77) = 4.43$, $p=0.039$) and Treatment ($F(2, 77) = 4.078$, $p=0.021$) variables. When we look post hoc, we could see that the differences are between CPF and VPA groups respect the control group ($p=0.007$ and $p=0.01$, respectively), with the exposed animals being the most delayed in emitting the first vocalizations, the control rats started with a mean of 6.2 ± 12.1 seconds, while CPF rats started around 38.1 ± 11.4 seconds and VPA rats started with a mean of 52.1 ± 11 seconds. No differences were found between CPF and VPA groups. In Sex variable, males with a mean of 18.2 ± 9.5 took significantly less time than females to emit the first call with a mean of 46.2 ± 9.2 ($p=0.036$). No differences were found in the Sex and Treatment interaction: $F(2, 77) = 0.693$, $p=0.503$.

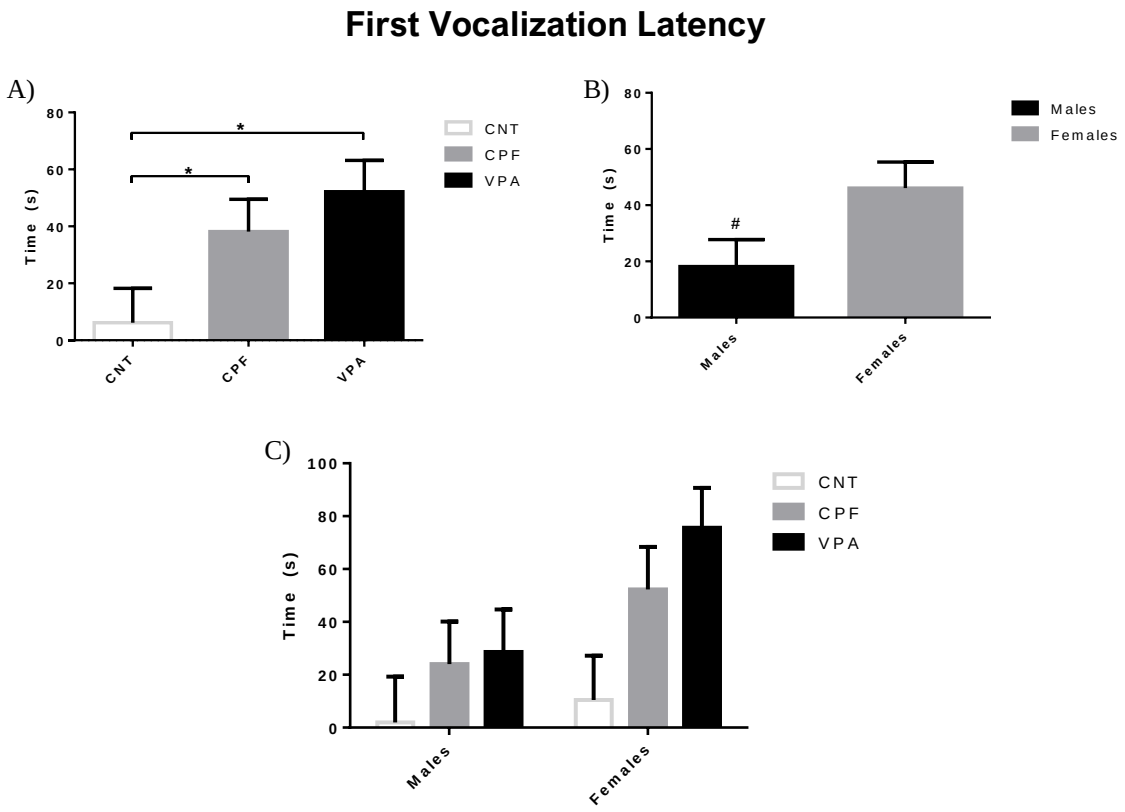

FIGURE 3: FIRST VOCALIZATION LATENCY GRAPHS. A) Shows how many time took to every treatment to emit their first vocalization: CPF and VPA groups significantly delayed the emission of the first USV respect control group (0.021<0.05). B) Shows a comparison of the latency between gender: males took significantly less time than females (0.036<0.05). C) No differences were found in the Sex and Treatment interaction: F (2, 77) =0.693, p=0.503.

When we looked at the data for differences in the first minute, we could see that the interaction of Sex and Treatment was significant (F (2, 76) =4.792, p=0.011), post hoc analysis indicated VPA males (p<0.028) and CPF females (p<0.024) were the ones who made fewer calls within their gender and treatment.

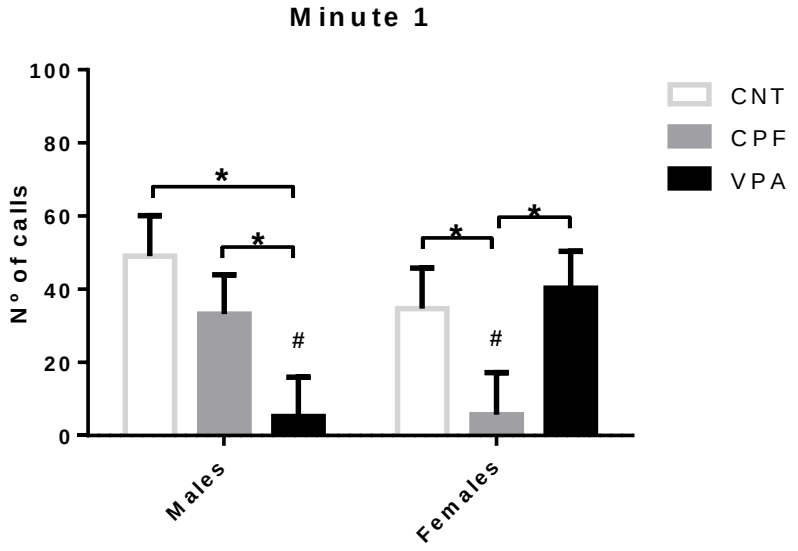

FIGURE 4: GRAPH OF USV DURING THE FIRST MINUTE. There were significant differences in the interaction of Sex and Treatment (0.011<0.05). Post hoc analysis showed control and CPF males emitted more USV than VPA males but CPF females emitted less USV than control and VPA females. In addition, VPA males made less USV than VPA females but were the CPF females which made significantly less USV than CPF males.

Second minute ANOVA results showed significance in the interaction of Sex and Treatment (F (2, 76) =4,241, p=0.018) again. Post hoc analysis indicated VPA males continued emitting less vocalizations than control males (p=0.008), but it was not significant respect CPF males (p=0.08). Also, VPA males emitted less vocalizations than VPA females (p=0.046). In contrast, it was the CPF females that emitted less vocalizations both than CPF males (p=0.026) and other female groups (p<0.027).

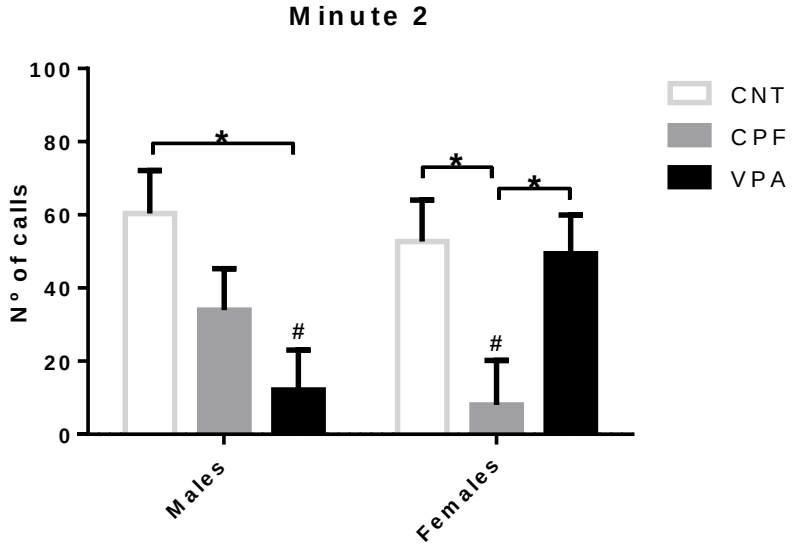

FIGURE 5: GRAPH OF USV DURING THE SECOND MINUTE. There were significant differences in the interaction of Sex and Treatment (0.018<0.05). Control males produced more USV than VPA males, which emitted less than VPA females. Within the female gender, it was the CPF that produced less USV, even less than CPF males.

In figure 6 we could see the significant differences disappeared (F (2, 79) =0.985, p=0.378). Although VPA males were the group which less USV emitted respect other male groups, with a mean of 22.6 ± 13.1 against 57.5 ± 14.1 of control and 57.8 ± 13.5 of CPF, and even respect VPA females with a mean of 51.9 ± 12.7, but not significantly. Females exposed (CPF: 50.4 ± 13.5; VPA: 51.9 ± 12.7) also seem to emit less USV than control females (75.9 ± 14.1).

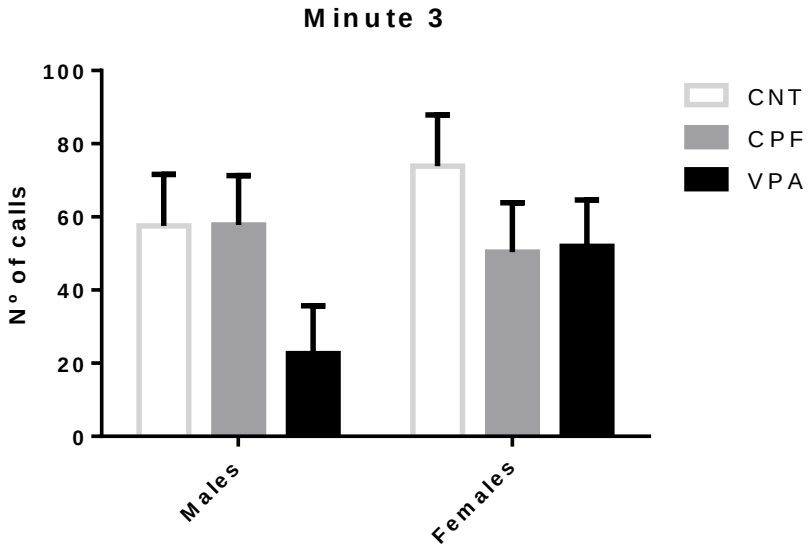

FIGURE 6: GRAPH OF USV DURING THE THIRD MINUTE. There were no significant differences (0.378>0.05).

4. DISCUSSION

Prenatal exposure to subtoxic doses of chlorpyrifos in the late gestational period has had effects on sociability and communication variables measured through the isolation from the dam protocol. Some studies have already used this developmental window, because is a period which corresponds roughly to the second trimester of pregnancy in humans. Even other studies have been used other routes of administration, they obtained similar results (Qiao et al., 2002; Venerosi et al., 2009; De Felice et al., 2015; Lan et al., 2017; Lan et al., 2019).

CPF intoxication is usually accompanied by a reduction in infant body length and weight (Smegal, 2000; Eaton et al., 2008; Lan et al., 2017), so we weighed daily the pups for assessing signs of intoxication, but no significant differences were found between treatments. Every treatment evolved in a progressive and similar way. Only on PND11 we found differences, where the VPA group weighed more than the control group, but this could be an anecdotic or punctual fact because it was not repeated. So, we could think

that actually this dose, proposed by several research groups (Qiao et al., 2002; Eaton et al., 2008), is a subtoxic dose that does not seem to inhibit AChE. So, the events that are going to be reported have acted in other ways.

The recording and analysis of the USV is a technique of low cost and high power and performance that has been proposed by many authors as an early preclinical marker of disease and presents certain characteristics similar to children crying (Venerosi et al., 2009; MacDonals & Brudzynski, 2018; Schewarting, Kisko & Wöhr, 2018). Some of these characteristics is the first vocalization latency, one of the main parameters that our laboratory team wanted to characterize and compare, due to the great importance of its function. Attending to the results, we can observe how the CPF and VPA groups take longer to emit the first vocalization with more than 32 and 46 seconds of difference respect to the control group respectively. Evidence that supports our hypothesis about a greater similarity of the CPF group with the ASD model. This was supported by similar evidences found by other rodent models of ASD (Venerosi et al., 2009; Schmeisser et al., 2012; Wöhr, 2014). The delay in the first vocalization latency could be explained by alterations in the dam-offspring bond, that is to say in the early social behavior of the pup. Venerosi et al., (2009) comment it is due to depressant effects of CPF. Even only in the exposed groups we have found offspring that have not issued any vocalization, malformations or troubles in the vocal tract are also discarded in a previous work of our laboratory group, non-published yet (Morales-Navas, non-published). So, this is an aspect that has to be investigated.

In addition, we found differences in the latency according to the sex, regardless of treatment. Females took longer to do the first vocalization, showing a sex-dimorphism, that other publications had already found (Venerosi et al., 2006). This is interesting if we take into account there is a higher prevalence of ASD in men (Werling & Geschwild, 2013).

The utterance of pups was measured by the number or rate of USV by minute (Venerosi et al., 2009) and not by the total number of USV because we found a big influence of latency, so the interesting fact was to see what happened during the different minutes. There was also a sexually dimorphic effect into the first and second minute. Females CPF emitted lower USV rate than CPF males in contrast with the VPA group, where males emitted the lowest rate into its treatment group. In the control group we also

can observe this, except in the third minute where the females in general increased the USV number, but no significant differences were found both into control group sex and any third-minute differences. This sex-dimorphism in the VPA group provides evidence of being a good animal model of ASD by environmental factors (Mabunga et al., 2015). In contrast with the principal effect found in CPF group that was also found in other procedures and ages predominantly in females, such as social recognition test at 4 moths age or light-dark exploration test at PND90 (Maggio & Whitney, 1985; Ricceri et al., 2003; Venerosi et al., 2006; Venerosi, Ricceri, Rungi, Sanghez & Calamandrei, 2010). The mechanisms through which this effect occurs are still not well defined, but some authors claim that CPF interferes in the maturation of sexually dimorphic neuroendocrine pathways in the developing of central nervous system (Leon-Olea et al., 2014; Venerosi et al., 2015). If we observed the results together, it was curious VPA males shown lowest latency than females even they were the ones that less USV emitted. That is, VPA males took less time to start emitting, but they emitted less, while females took more time. An observation that we had not yet found in the literature.

When we looked at the results of the third minute, we not found significant differences, because there was a generalized increase in the number of USV. We wanted to be sure that the difference in the USV number was not due to the effect of the first vocalization latency, that is, once all the rats started emitting, they had the same USV rate, but we rejected this attentional deficit hypothesis for two reasons. The first was the VPA males had a relatively small latency and, nonetheless, they were the group that least USV emitted. The second reason was that the increase, that as we have already mentioned, was general and we also found it in the control group. As we can see the female controls increase from 52 to 74 calls. Thus, probably the effect has more relation with the fact that procedure itself supposes an aversive situation for the animal (unknown environment, loss of mother protection, change of temperature, etc). There are several experimental evidences supporting USV are also emitted under nonsocial keys but as indicators of the emotional or motivational state of neonate rodents (mood, pain, stress, environmental conditions, appetitive or aversive situations, etc) (Hofer, 1987; Hofer, Brunelli & Shair, 1993; Portforts, 2007; Venerosi et al., 2009; Simola & Granon, 2018). This is in accordance with data that showed transections through or just anterior to the hypothalamus completely blocked social isolation-induced vocalizations of infant rats, but not ultrasonic vocalizations induced by hypothermia during the isolation (Middlemis-

Brown, Johnson & Blumberg, 2005; Muller & Shair, 2016). Other authors also have found that deactivation of the cholinergic basal forebrain affected calls during a fear conditioning procedure, but no on isolation-induced vocalization (Ricceri, Cutuli, Venerosi, Scattoni & Calamandrei, 2007; Muller & Shair, 2016).

One of our limitations is the lack of a developmental functional battery that would have allowed us to study the possible differences between the treatments in the development to ensure that the evidences found are due to other mechanisms than AChE inhibition. Another interesting modification is the analysis of the duration and peak frequency of the USV. These are other USV parameters with similar characteristics to the crying of children (Venerosi et al., 2009). So more research is needed, because, although the use of USV in rodent research is increasingly widespread, there are still few studies on the effect of CPF in this procedure. Mice are the main species used for this purpose instead of rats that have complex patterns of social behavior. This is due to the great genetic variability presented by different strains of mice (Schneider et al., 2008). Thus, we have not found any study in rats exposed to a subtoxic dose of CPF in GD12-15 that has analyzed its effect in USV on PND7. And even if some studies found evidences of ASD signs, they have not been robustly replicated, probably due to methodological differences between labs (Williams & DeSesso, 2014; Lan et al., 2017).

5. CONCLUSIONS

There is a remarkable sex-dimorphism effect in the exposition, both CPF and VPA groups during the late gestational period, even in subtoxic doses. However, both sexes show more depressed behavioral patterns of social communication than the control group without signs of intoxication, so other mechanisms to AChE inhibition must be mediating the effects. More research is necessary to understand why the main effect of VPA occurs in males, consequently with the existing evidence of validated animal model, and the main effect of CPF occurs in females.

6. CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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