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Neurotoxicological effects of chlorpyrifos prenatal exposure in adolescent rats

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Summary

Autism spectrum disorder (ASD) aetiology contemplate genetic, environmental and other risk factors. Pesticides and specifically chlorpyrifos (CPF) are well-known environmental risk factors which can produce molecular changes that are related to the development of ASD.

In this project, the purpose was to analyse interaction of genetics and environmental risk factors, with 49 adolescents Sprague Dawley rats. In order to do that, it was studied a model of CPF prenatal exposure (1mg/kg/mL/day, GD 12.5-15.5), *fmr1*-KO model and *fmr1*-KO model prenatally exposed to CPF. Both RT-qPCR of the hippocampus and three chambers Crawley test (CCT) were analysed for all the subjects and finally results were correlated.

Results proved that *fmr1-KO* and CPF-*fmr1*-KO (CPF-KO) conditions affect sociability as well as genetic expression with a correlation between both variables. There is an additive/suppressive effect of both factors for CPF-KO rats, reflected in a decrease of time spent with S1 and increase of 5-ht2c and *kcc1* expression. Moreover, *fmr1*-KO demonstrated to be a good ASD model which shows a reduction in sniffing and social efficiency and SNI ; s well as a rise in *grin2c* expression. Finally, some sociability parameters (SOCS1, SI and OBSF3TIMES2) correlated negatively with gene expression of *gad1*, *nicoα7* (for SOCS1, SI) and *gaba2* (for OBSF3TIMES2).

Resumen.

La etilogía del trastorno del espectro autista (ASD) contempla factores de riesgo genéticos, ambientales y de otro tipo. Los pesticidas, y específicamente el clorpirifos (CPF), son factores de riesgo ambientales que pueden producir cambios moleculares relacionados con el desarrollo de ASD.

En este proyecto, el propósito fue analizar la interacción de la genética y los factores de riesgo ambientales, con 49 ratas Sprague Dawley adolescentes. Para ello, se estudió un modelo de exposición prenatal a CPF (1 mg/kg/mL/día, GD 12.5-15.5), el modelo *fmr1*-KO y el modelo *fmr1*-KO expuesto prenatalmente a CPF. Tanto la RT-qPCR del hipocampo como la prueba de Crawley de tres cámaras (CCT) se analizaron para todos los sujetos y finalmente se correlacionaron los resultados.

Los resultados mostraron que las condiciones *fmr1*-KO y CPF-*fmr1*-KO (CPF-KO) afectan la sociabilidad y la expresión genética con una correlación entre ambas variables. Existe un efecto aditivo / supresor de ambos factores para las ratas CPF-KO, que se refleja en una disminución del tiempo pasado con S1 y un aumento de la expresión de 5-*ht2c* y *kcc1*. Además, *fmr1*-KO demostró ser un buen modelo de ASD que muestra una reducción en el olfateo, eficiencia social y SNI; y un aumento en la expresión de *grin2c*. Finalmente, algunos parámetros de sociabilidad (SOCS1 SI y OBSF3TIMES2) correlacionaron negativamente con la expresión génica de *gad1*, *gaba2 (para* SOCS1 y SI) y *nicoα7 (para* OBSF3TIMES2) .

CONTENTS:

1.-INTRODUCTION

1.1. Autism spectrum disorder (ASD) pathology

Autism spectrum disorder (ASD) is a neurodevelopmental broad-spectrum condition which appears very early in the childhood (Nitschke et al., 2020). According to DMS-5 (Diagnostic and Statistical Manual of Mental Disorders), ASD diagnosis consists in a notorious clinical profile of impaired communication/interaction and repetitive patterns of behaviours, interests, and activities (Nitschke et al., 2020). Associated symptoms could include disruptive impulse-control, unpredictable learning, unusual responses to sensory input, executive dysfunction, anxiety, attention deficit, hyperactivity and can be accompanied by intellectual impairment (Grove et al., 2019; Paudel & Singh, 2021). Along with the traditional DSM-based diagnosis, the state-of-art machine learning technologies have recently enabled the identification of ASD biomarkers in pregnant women as a promising progress for ASD diagnosis (Caly et al., 2021). On the contrary, some of the most accepted behavioural tests for ASD in animal models include marble burying (compulsivity or anxiety), T-maze spontaneous alternation (learning), ultrasonic vocalization (USV) (communication, interaction), three-chambered social preference test (social interaction and reaction to novelty) or open field test (motricity and anxiety) (Chang, Cole & Costa, 2017), amongst others.

Notwithstanding ceaseless scientific research on this field, ASD aetiology remains unsolved, pointing out a combination of different genetic and environmental factors (Nitschke et al., 2020; Grabrucker, 2013). ASD is speculated to show up in the womb, where also neuroanatomical changes take place, affecting neurodevelopment (Paudel & Singh, 2021). Experimental observations in diagnosed children and ASD-like rats brought to light an excess of neurons in the prefrontal cortex, brain overgrowth (cortical expansion), hippocampus and ventricles asymmetry and megalencephalic brain (Caly et al., 2021). Neurochemical alterations also take place in ASD, including deregulated signalling pathways or abnormalities in neurotransmitter system which directly affect behaviour (Modabbernia, Velthorst & Reichenberg, 2017)

ASD treatment chiefly consists of behavioural therapy. Conversely, drugs are still on preclinical studies, being antiepileptics the only one used on some ASD patients

(Cetin et al., 2015). A potential treatment for ASD could be using drugs that inhibit GABAergic system (i.e. arbaclofen) or NMDA antagonists (i.e. memantine) (Brondino et al., 2016; Cetin et al., 2015). Another approach could be abrogating IL-17A activity, a proinflammatory cytokine involved in ASD development (Wong & Hoeffer, 2018). Recently, Teijeiro et al. (2021) discovered that the known drug Digoxine can inhibit IL-17 activity without almost secondary effects in mice. Therefore, this drug stand to be used in a near future for a broad variety of inflammatory disorders including cancer, obesity or ASD.

Since ASD was taken to the clinics in the 1960s, its global prevalence has steadily increased from 0.041 to 1.68% (Caly et al., 2021). Its prevalence is soaring concerningly in developed countries, especially in North America, being four times more abundant in men than women (Nitschke et al., 2020). In United States, the incidence has increased 32% since 2014. The sharping rise of this pathology is unclear, as it could be due to changes in diagnosis criteria, wider access to diagnosis or increment of ASD risk factors (Cybulski et al., 2021; Davidovitch et al., 2021; Caly et al., 2021; Berg et al., 2020; World Health Organization, 2019; Hansen, Schendel & Parner, 2015). Substantial short-term increases in ASD prevalence may not be attributed solely to genetic factors and/or improved diagnostic criteria. There is now a consensus that multiple genetic loci combined with environmental risk factors during critical periods of neurodevelopment influence ASD susceptibility and symptomatology, a topic of interest in the ongoing investigation (Chaste et al., 2012).

1.2. ASD risk factors

1.2.1. Genetics

It is noteworthy that genetic burden is bound to ASD development. Not only does heredity, but also *de novo* mutations underlie ASD aetiology. A total of more than 1.000 genetic risk variants have been linked with ASD development, for instance, genes that encode neuronal regulator factors (*negr1* and *ptbp2*), calcium-binding protein (*cadps*), independent-calcium channels (*kcnnn2*) chromatin remodelers (*kmt2e*), post translational modificators (*macrod2)* or synaptic genes (*nlgn-nrxn-shank*) (Grove et al., 2019). Other genetic mutations have been reported for: *dpp6, fmr1, scn2/4a, grin2b, wnt3, park2, foxp1/2, arid1b, ash1l, chd2/13, nrxn, nlgn1/3, nlgn4x, reln, kmt2a, chd8, dyrk1a, pogz, cntn3, ptchd1, shank3, syngap1,*

cntnap2, cdh8, pten and others (Li & Pozzo, 2019; Chaste et al., 2012; O'Roack et al., 2012; Li et al., 2010). These genes do not act independently, they interact among them triggering deregulation of signalling pathways for ERK/MAPK, Wnt/β-catenin or PI3K-Akt-mTOR pathway (Gazestani et al., 2019; Vithayathil, Pucilowska & Landreth, 2018; O'Roack et al., 2012; Chen et al., 2015). It must be also mentioned, the importance of genes that encode neurotransmitters receptors which are highly altered in ASD, including GABAergic, serotonergic, dopaminergic, acetylcholinergic, chathecolaminergic or glutamatergic systems (Caly et al., 2021; Cetin et al., 2015) (see Appendix 2). As an outcome, there is a net effect of overexcitation, a key pattern in the spectrum (Selimbeyoglu et al., 2017).

Most common ASD genetic-related syndromes in humans include Fragile-X syndrome (FXS), Rett syndrome, foetal anticonvulsant syndrome or cytogenetic abnormalities (Chaste et al., 2012; Coghlan et al., 2012). Slick transgenic models can ape these well-known genetic disorders; with special interest on FXS model. Fragile X Mental Retardation 1 gene (*fmr1*) produces Fragile X mental retardation protein (FMRP), which plays a pivotal role in synaptic plasticity (Zhao & Bhattacharyya, 2018). Inactivation of this gene by mutation gives rise to FXS, characterized by a clinical-ASD picture (decreased social interaction, anxiety, hyperactivity, impaired sustained attention, intellectual disability and difficulties in inhibition response) and is present in 2-6% of ASD population (Richter & Zhao, 2021; Zhao & Bhattacharyya, 2018). Therefore, a *fmr1*-knockout (KO) rat is likely to be a good preclinical model to study ASD pathology. This animal model simulates FXS, but instead of being a CGG expansion and methylation as happen in humans, there is a deletion of *fmr1* gene (Richter & Zhao, 2021). Other genetic models to mimic ASD could include *tsc2* deletion, *disc* mutant, *btbr* T^{+}*tf/J* or the reelin deficient model (Chang, Cole & Costa, 2017; Rosset et al., 2017; Marchetto et al., 2010).

1.2.2. Environmental factors

A growing body of research shows that environmental factors, especially those which have a detrimental effect the developing foetus, play an important role in ASD (Alampi et al., 2021).

In this range, it could be included: parental age, maternal diabetes, birth complications, pre-partum drug exposure (ie. valproic acid, misoprostol, thalidomide, antidepressants) and toxics exposure as could be phthalates, polychlorinated biphenyl, or pesticides such as organochlorines (OCs) (Alampi et al., 2021; Chaste et al., 2012). On the other hand, other chemical compounds allegedly related to ASD development, include bisphenol A, lead, triclosan, mercury or organophosphate (OP) pesticides (Alampi et al., 2021). It is by no means certain the downstream signalling pathway of these compounds, neither how do they trigger ASD in humans; albeit thriving research is being done (Pérez-Fernández, 2020; Morales-Navas et al., 2020).

As a matter of fact, environmental factors are important in signalling pathways, epigenetics, toxicology and even in our microbiota composition. Being affected all these features in ASD. (De Angelis et al., 2013; Rianda et al., 2019; Olsen & Dick, 2019; Fattorusso et al., 2019; Chaste et al., 2012).

1.3. Pesticides.

Nowadays, there is a great variety of pesticides for controlling a wide range of pests (Richardson et al., 2019). Worldwide pesticide usage approaches almost 8 billion pounds (3.6 billion kg) of active ingredient per year, and this quantity is expected to increase (Richardson et al., 2019).

Pesticides are classified based on their chemical or physical properties, origin, and target (Berg et al., 2020; Pérez-Fernández, 2020). Historically, first pesticides used by humankind dates before the 19th century, being sulphur the first one. Then, toxic metals like arsenic or natural products as pyrethrum or rotenone arrived to the market. Closer our decade, synthetic organic chemicals were launched into the market, being the most well-known modern pesticides as carbamates, OCs and OPs (Richardson et al., 2019).

The neurotoxicological effects of these compounds have been systematically proved and the concern relating this topic is rising with its widespread usage, being present in our food and surround (Richardson et al., 2019). Pesticide's health effects are rising owing to the empirical outcomes of their intake, They are estimated to produce over 3 million poisonings and more than 200.000 defunctions per year (Pérez-Fernández, 2020). This untoward effect is due to a similar mode of action

for both target and non-target organisms, with special importance in the case of OCs, OPs, and pyrethroid pesticides (Richardson et al., 2019). The exposure outcome ranges from short-term to chronic impacts, including various cancers, psychiatric problems, birth defects, infertility, and endocrine disruption (Albaho, Ahmed & Devi, 2017).

Among the most widely used insecticides are the OPs pesticides, which include Chlorpyrifos (CPF), parathion, and diazinon (Berg et al., 2020). CPF lousy reputation led to its partially control in the last century. The current authorisation for CPF use expired in Europe in 2020, whereas the fate of CPF regulation is still uncertain in the USA (Macirella et al., 2020). Due to the lack of awareness CPF is still one of the most widely used OP pesticides worldwide (Berg et al., 2020).

1.4. CPF metabolism.

CPF is a neutral organic phosphorothionate which belongs to non-volatile OP. Its onset on market was in 1965, catalogued as a broad-spectrum pesticide, which is obtained from thiophophoricacid. (Rathod & Garg, 2017; Ellison et al., 2011). Supplementary data regarding physical-chemical properties and toxicokinetic are available in APPENDIX1.

CPF intake ways include oral ingestion (enteric system), inhalation (respiratory absorption) or in lower amounts dermal absorption (Rathod & Garg, 2017; Eaton et al., 2008). As it is liposoluble, it can be accumulated in the fat (Fig.1). Most of its bioactivation takes place in the liver, while detoxification rely on the liver and plasma (National Center for Biotechnology Information, 2021). P450 enzyme oxidizes CPF to an intermediate form (phosphooxythiiran intermediate), which afterwards, undergoes either (1) oxidative desulfurization, producing the CPF-oxon (CPO), or (2) dearylation (oxidative ester cleavage) with paraoxonase enzyme (PON), forming 3,5,6-trichloro-2-pyridinol (TCPy) and diethylthiophosphate (DETP). At the same time, PON can modify CPO, generating TCPy and diethylphosphate (DEP) (Smith et al., 2011) (Fig.1). The CPO metabolite is the most reactive which reaches its blood peak 1-3h after ingestion. The oxonic form can reach the brain, but also can be found in liver, kidney or fat (Rathod & Garg, 2017; Eaton et al., 2008).

Products of metabolism of CPF (TCPy, DETP and DEP) are inactive forms which usually are the ones excreted (Smith et al., 2011). Once metabolized, most of the excretion is done commonly within 48-72h after ingestion, trough urine (84%) or milk (1%). TCP alone or in its glucuronide or sulfate form is a biomarker commonly used to analyse CPF exposure in urine. Nevertheless, CPF can be excreted untouched via feces (5%) (Rathod & Garg, 2017) (Fig.1). Excretion half-life is dependent on sex (being longer this time for females), dose quantity and via of administration (National Center for Biotechnology Information, 2021). CPF metabolism share similarities in the animal kingdom with small variations in half-life, hence animal models offer a prospering approach for toxicological studies.

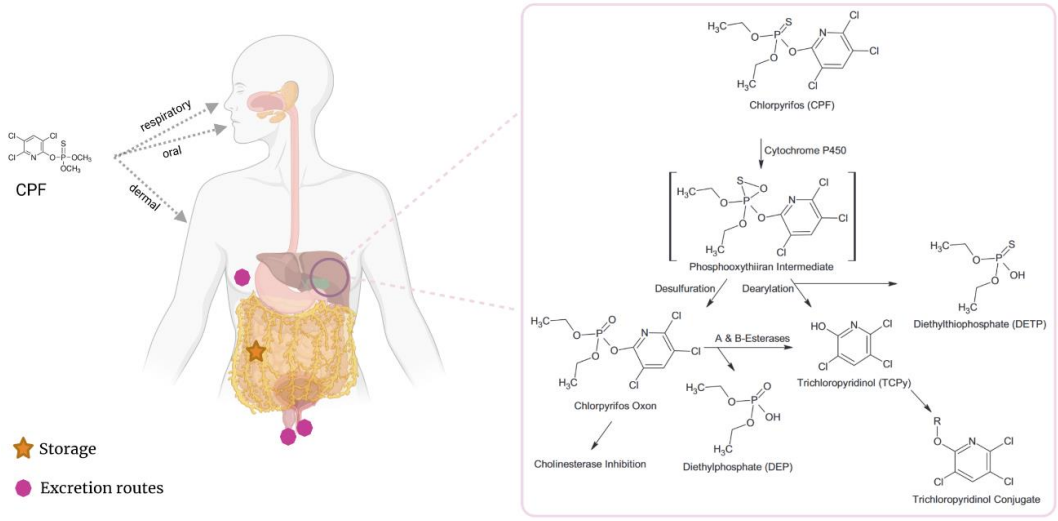

Figure 1. Intake ways, enzymatic metabolism, storage and excretion routes of CPF.

1.5. CPF toxicology.

CPF is a neurotoxic agent, that is especially effective in early stages of development (prenatal), as desintoxication enzymes (paraoxonase and carboxylesterase) remain lower in new-borns (Eaton et al., 2008). It is not considered teratogenic but nonetheless, some correlations were found with lung and colorectal cancer (Eaton et al., 2008). CPF exerts undesirable effects chiefly on central nervous system (CNS), but also cardiovascular and/or the respiratory system.

The canonical mechanism of action of CPO is irreversible inhibition of AChE or pseudocholinesterase (BuChE) (Fig.2.1). AChE underlies the hydrolysis of acetylcholine (ACh). Therefore, if AChE activity is missing, ACh accumulates in the synaptic cleft and overstimulates neurons with ACh receptors, which includes nicotinic and muscarinic receptors (National Center for Biotechnology

Information, 2021; Eaton et al., 2008). As a result of CPO toxicity, can be also altered: oxidative metabolism and genotoxicity (Fig.2.2); endocrine system (Fig.2.3); neuronal growth and neurotransmission (Fig.2.4); signalling pathways and neurotransmitter system (Fig.2.5) (Tadee, Mahakunakorn & Porasuphatana, 2020; Rathod & Garg, 2017).

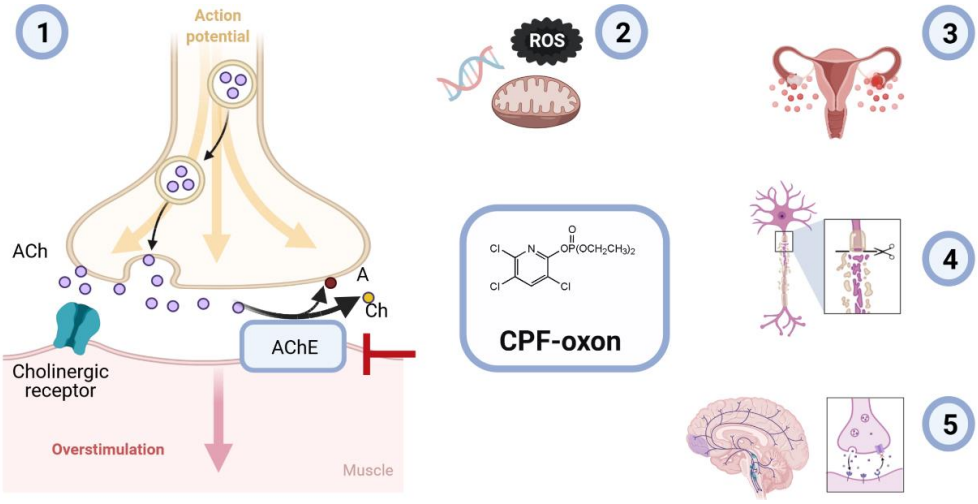

Figure 2. Methods of action of CPO. 1) Canonical inhibition of AChE activity, which produces accumulation of ACh in the synaptic cleft. Non canonical outcomes of CPF exposure include for the categories above mentioned: 2) Oxidative metabolism and genotoxicity: DNA damage, decreased cell viability and differentiation, inhibition of mitochondrial oxidative phosphorylases, mitochondrial mismatch, reduced glutathione (GSH), and superoxide dismutase (SOD); whilst increased glutathione peroxidase (GPx) and paraoxonase 1 activity; 3) Endocrine system: It is hypothesized that CPF acts as an endocrine-disrupting chemical: it modulates vasopressin, weakens estrogen and inhibits testosterone and estradiol 4) Neuronal growth and neurotransmission: CPF inhibit a myelinating enzyme, neuropathy target esterase (NTE), alters axogenesis and functioning of neurotrophic signalling cascade and produces aberrant synaptic activity 5) Signalling pathways and neurotransmitter system: CPF alters patterns of lipid signalling, CREB phosphorylation or affects cell signalling mediated by adenylyl cyclase (AC). Moreover, it can modulate transcription factors, gene expression and can have a direct action on proteins. It can also be affected the serotonergic, dopaminergic, GABAergic or endocannabinoid) (Ubaid et al., 2021; Odetti et al., 2020; Tadee, Mahakunakorn & Porasuphatana, 2020; Eaton et al., 2008)

Surprisingly, low CPF doses (around 1mg/kg/day) involve non-cholinesterase mechanisms (NChEI). However, NChEI doses of CPF have been related to specific alterations in different components of the most important neurotransmitter systems, such as serotonergic (Aldridge et al.[1], 2005; Raines, Seidler, & Slotkin, 2005), GABAergic (Pérez-Fernández, 2020; Sánchez-Amate, Flores & Sánchez-Santed, 2001; Sánchez-Amate et al., 2002), dopaminergic (Pérez-Fernández, 2020; Aldridge et al.[2], 2005; Slotkin & Seidler, 2007; Jett et al., 2001; Pope et al., 1992; Moser et al., 1998), or even endocannabinoid (Carr et al., 2017; Carr et al., 2014; Carr et al., 2013; Carr, Borazjany & Ross, 2011; Carr et al., 2001). There are noticeable molecular effects of NChEI doses, but still

a lot of missing information, so studying effects of these doses is extremely important. The reason why subtoxic CPF doses have been overlooked for long time is because they do not produce dramatic acute effects. Human CPF intake is usually closer to NChEI doses, which have been recently correlated with cognitive alterations, psychiatric disorders, and neurodegenerative diseases (Eaton et al., 2008).

CPF neurotoxicity is upholded by the identification of neurological symptoms along with changes in cognitive and psychomotor function (Moser et al., 2005). It is considered a risk factor of ASD, attentional deficit and hyperactivity disorder (ADHD), Alzheimer's disease, Parkinson disease, amyotrophic lateral sclerosis, anxiety, or depression (Sánchez-Santed, Colomina & Herrero, 2016). A critical period in the development of this pathology is pregnancy and have been reported links between prenatal CPF exposure and ASD diagnosis in human (Silva, 2020; von Ehrenstenin et al., 2019). But this is not a fact clearly stablished, as contradictory results have been obtained for CPF exposure along development (Alampi et al., 2021; Berg et al., 2020; Guo et al., 2019; Eskenazi et al., 2010; Rauh et al., 2006). However, it appears to be a growing body of empirical evidence of greater sensitivity during pregnancy, something that makes sense looking at epidemiological studies in humans. According to Silva (2020) NChEI doses administered prenatally to different animal models, including rodents, show a ASD-like behaviour. The studies in mice by Lan et al. (2017; 2019) proved that the GD12 period appears to be especially sensitive to CPF and observed an association with ASD behaviour. This study was performed in mice, although rats are a better model for social traits (Ellenbroek & Youn, 2016). Our laboratory was the first to show similar ASD-like effects in rats exposed to CPF during this period (Morales-Navas et al., 2020), as well as being the first to characterize the metabolic profile of these animals (Abreu et al., 2021). However, the influences that such exposure may have on genetically vulnerable models, such as *fmr1*-KO, have not been studied yet.

NChEI doses of CPF are still under-explored and in view of the fact that some prenatal exposure studies point out the existence of neurobehavioral changes, it must be studied to reveal hallmarks of alternative toxic pathways.

2.-HYPHOTESIS AND OBJECTIVES

2.1. Hypothesis

This research is a step forward on the convoluted interaction of environmental and genetic ASD risk factors, CPF and *fmr1*-KO respectively in adolescent rats. Allegedly, the sum of both factors could strength the ASD-like profile (Bai et al., 2019).

Coming along with behavioural alterations, it is predictable to take place the alterations of different components from the main neurotransmitter systems at gene expression level.

2.2. Objectives:

Taken all together, the general objectives are:

1. Compare social interaction of ASD models and WT adolescent rats.

2. Characterize and compare genetic expression of adolescent rat hippocampus, with different models of ASD susceptibility and WT.

3. Correlate social interaction, genetic analysis and ASD risk factors.

3.-METHODOLOGY

3.1. Experimental animals

3.1.1. Rats description

Experiments were performed in F2 generation (N=49) of WT and *fmr1*-KO Sprague Dawley male adolescent rats. Experimental rats were weaned and accommodated in groups of 4 of the same sex in polycarbonate cages at PND21: 26.5 (width)×18 (height)×42 (length) cm, with controlled room temperature and humidity, 22±2ºC and 50±10%, respectively. Moreover, circadian rhythm was regulated under a 12/12 h light/dark cycle (light turn on at 8pm). Food and water were available *ad libitum*. Behavioural tests were performed at the nocturnal phase when rats were awakened.

The experiment belongs to the project ES040130002260 and was conducted in accordance with the Spanish Royal Decree 53/2013, the European Community Directive (2010/63/EU) for animal research and comply with the ARRIVE guidelines for animal research. All the experiments described in the present

manuscript have been approved by the University of Almeria Animal Research Committee.

3.1.2. Obtention of F2 generation

The population of study consisted of F2 males *fmr1*-KO and wild-type (WT). F0 generation consisted of 15 WT female and 5 hemizygote *fmr1*-KO male rats, obtained from SAGE Labs Inc. and Janvier, respectively (3 months of age). It must be stated that F0 rats, after acquisition, were allowed for 2 weeks of habituation to our facilities previous mating. Male and female in proestrus/estrus were left in a cage for 24h to ensure copulation. After checking presence of sperm in the female, fecundated rat was isolated in a cage and this was considered as gestational day (GD) 0. F0 offspring (postnatal day 0, PND0) gave rise to F1 consistent of *fmr*1-KO heterozygotes females (50%) and WT males (50%) (Fig.3). On PND21, pups were weaned, and separated by sex into groups of 4 rats. Once this F1 animals were > 3 months-old, they were mated (same process as previously described) and pesticide exposure was randomly performed on half of pregnant F1 mothers from each group as described in Figure 4. All the groups underwent injection of CPF vehicle to eradicate differences amongst treated and untreated groups (Fig.4). Then, F2 consisted of heterozygous females *fmr1*-KO (25%), hemizygous males *fmr1*-KO (25%), WT males (25%) and WT females (25%). (Fig 3). Only males from F2 were used for this set of experiments as this sex is more prompt to develop the pathology in humans (Weling & Geswind, 2013). Therefore, F2 resulted in 4 groups of male rats disposed as the following: 1) WT+vehicle; 2) WT+CPF; 3) *fmr1*-KO+vehicle; 4) *fmr1*-KO+CPF.

All the experiments were performed with triple blind, only knowing each rat condition once the experiments and analysis were finished.

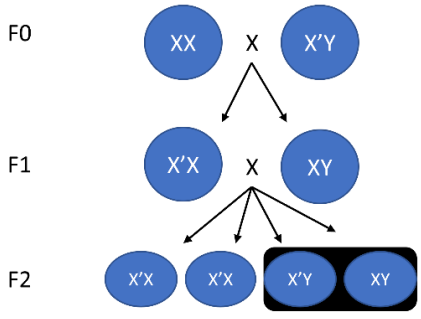

Figure 3. Animal matting and obtention of the consecutive generations. Only those animals belonging to the F2, included in the black square were used in the analysis of this project.

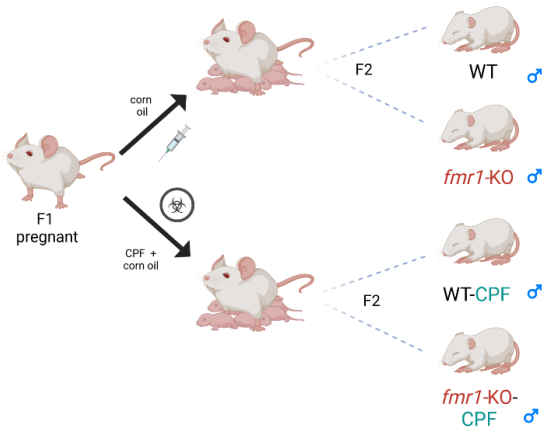

Figure 4. Groups of study. F1 pregnant rats were injected half with CPF+corn oil, whilst the other half only corn oil from GD12.5 to 15.5. Following that, males from each group litter conformed F2, with 4 groups well distinguished.

3.1.3. Toxic agent

CPF exposure was performed by a subcutaneous injection of NChEI dose (1mg/kg/mL/day) of CPF [O, O-dietil O-3,5,6-tricloropiridina-2-il fosforotioato PESTANAL®, from Sigma-Aldrich (ref. no. 45395; purity > 99%)], diluted in corn oil as vehicle. This vehicle was injected to all the rats. CPF dose was chosen not to inhibit ChE during development (Pérez-Fernandez et al., 2019; Yan et al., 2012; Venerosi et al., 2009) but to produce specific neurological alterations in adolescent rats with the purpose of generating ASD-like behaviour (Morales-Navas et al., 2020). Half of F1 mums were randomly injected from GD12.5 to 15.5. GD12 seems to be a sensitive time span for CPF exposure, as previously reported in both mice (Lan et al., 2017; 2019) and rats (Morales-Navas et al., 2020); but in this case, this was dependent of a vulnerable genetic background (*fmr1*-KO) generally linked to ASD-like behaviours. All the structured schedule of rat interventions, behavioural and biochemical analysis to which rats were subdued are indicated in Figure 5.

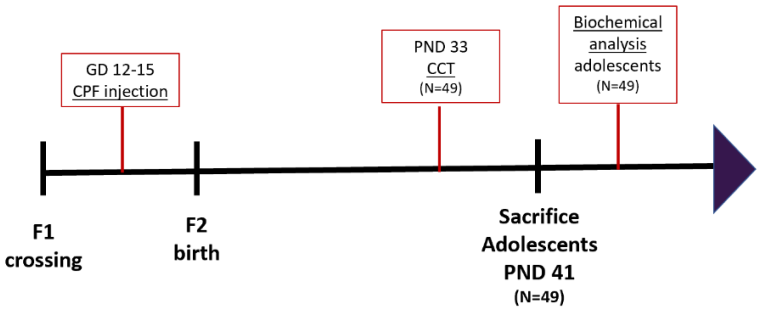

Figure 5. Experimental timeline. Prior to birth, half of F1 mothers were injected CPF and F2 was the generation in which all the task and biochemical analysis were done. For F2 it is indicated each proof in the day it was done and also the number of animals affected in each step.

3.2. Behavioural test

3.2.1. Crawley's sociability and preference for social novelty test. Three chambers Crawley test (CCT). PND33 N=49

This apparatus allows detection of direct social behaviour as the rats are given the option of interacting with novel rats or objects (Fig. 6). It is a measure of three behavioural conditions: habituation, sociability, and reaction to social novelty, parameters generally affected in ASD (Yang, Silverman & Crawley, 2011).

CCT consists of a Plexiglas customed rectangular, three-chambered box, with one central and two others equal-in-size chambers at each side. Each chamber with a dimension of 30x98x50cm was separated by two Plexiglas walls of 50cm, with one gate of 10x10cm in both walls, allowing rat movement from one chamber to the other. Rats were driven to the experimental room one hour before the experiment started. During the first phase (habituation phase), experimental rat was placed in the central chamber and their motor behaviour was monitored for 5min with all the gates closed (Fig.6). Following this, in a second phase (social phase), one unknown animal (namely Stranger 1, or S1) was introduced in a grid cage (23x15x23 cm) placed in the right lateral chamber. Gates were opened and experimental rat's exploratory behaviour was recorded for 10min (Fig.6). After this period, third phase (reaction to novelty) started. Then it was introduced another unknown rat (S2) in a grid cage situated in the left lateral chamber, maintaining the S1 on its previous place. Experimental rat could freely explore the paradigm for other 10min (Fig.6).

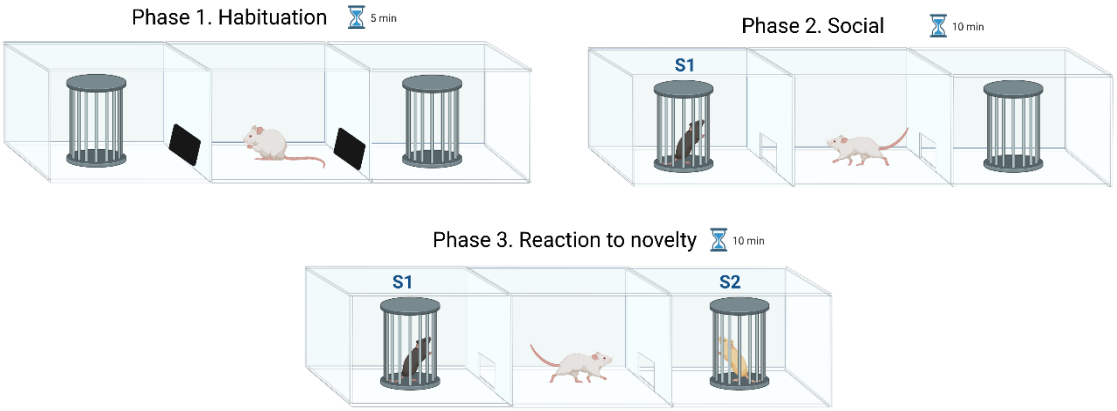

Figure 6. Experimental designing of three chambers Crawley test (CCT) with all the phases.

Concerning social behaviour, it was recorded the time spent in each compartment (s), sniffing time to each grid (s) and social efficiency (ratio). Social efficiency was a combination of time spent with S1 and direct interaction. On top of that, social index (SI) and reaction to social novelty (SNI) were included in the analysis and were calculated following the formulas: $SI = \frac{S1-empty}{S1+empty}$ and $SNI = \frac{S2-S1}{S2+S1}$

70% ethanol was used to polish chambers in between subjects. Data was analysed by the video tracking program Etho-Vision v3.1 (Noldus). Animals' order was counter-balanced concerning TREATMENT condition (CPF exposed rats made the test during even sessions, while control rats completed it during odd sessions). This experimental procedure was carried out in the time fram between 9 and 13 am under dim-light conditions. Temperature and humidity were set as those previously described for the home-room condition.

3.3. Biochemical analysis

3.3.1. Sacrifice. PND41

Rats were sacrificed during adolescence on PND41. For brain extraction, rats were deeply anesthetized with inhalation of isoflurane in a chamber for at least 3min and rapidly decapitated. The entire brain of adolescents was instantaneously removed and dissected in frontal cortex, hippocampus, hypothalamus, and cerebellum.

Brain structures were instantly flash frozen at -80ºC, to avoid RNA and protein degradation. Finally, proper cleaning of surfaces and materials from sacrifice was done via autoclave or with RNAse treatment. Only samples from hippocampus were used for the present set of experiments.

3.3.2. Genotyping by PCR

After each sacrifice, ear samples were collected to isolate gDNA using a column-based method (GeneJET, Fisher Scientific). Isolated and purified gDNA quantity and quality (260/280 ratio) were checked with Nanodrop©. For PCR, 10ng/uL of gDNA were used along with Taq Nzytech Master Mix, RNA-free water, and primers. Primers' sequences were Forward:5'-tggcatagaccttcagtagcc-3' and Reverse: 5'-tatttgcttctctgaggggg-3' (Hamilton et al., 2014). PCR conditions were the following: 10min at 94ºC, 35 cycles of 1 minute at 94ºC, 1 minute at 59ºC and another minute at 72ºC, and 10 minutes at 72ºC. Following the PCR, samples were

charged in an agarose gel and electrophoresis begun. WT samples expressed at 400bp while KO did it around <300bp.

3.3.3. Gene expression by reverse transcription quantitative polymerase chain reaction. RT-qPCR

a) RNA extraction

Frozen hippocampus samples stored at -80ºC were used for RNA extraction. Initially, frozen samples were added to a tube containing 1mL of Trizol reagent (Invitrogen), all on ice. Subsequently, a Heidolph © DIAX 900 homogenizer was used for each sample for a period of 8s, 3 times, all on ice. Next, the already homogenized content was transferred to an eppendorf and centrifuged at 12000G for 10 min at 4ºC. Once the centrifugation finished, 600 µL of the supernatant were collected into a new eppendorf, left to rest for 5min at room temperature. After this period, 100µL of 1-Bromo-3-chloropropane were added which is stirred manually for a time of 15 seconds, and let it rest for 10min at room temperature. Next, it was centrifuged at 12000G for 10min at 4ºC and 150 µL of the upper supernatant (RNA) were collected, passing this to a new eppendorf where also were added 500 µL of isopropanol. Then, it was homogenized and left 7 min at 25ºC. It was centrifuged at 12000G for 10min and once finished, the supernatant was discarded and 1mL of cold 75% ethanol added, homogenized and vortexed. In addition, it was centrifuged at 7500G for 5min at 4ºC. After the latter, everything was discarded except the pellet, which was left to dry on ice with the lid open for 7 min. 50 µL of RNA-free water were added to eppendorfs and then they were placed in a dry bath at 57ºC for 12 min (homogenizing every 2min with a pipette). The samples were left to rest for 2min on ice and centrifuged at 8000G for 40s at 4ºC. In this way, RNA was obtained.

After this, RNA was diluted 1:100 in nuclease-free water, which was used for fluorescence quantification (Fluorescence Qubit ©). On the other hand, 3µL of RNA were transferred to a new tube that was used to check RNA purity by NanoDrop©. In parallel, samples were run on a 1.5% agarose gel electrophoresis in MOPS and revealed by incubation with 1/50000 GelRed. Agarose gel allowed 28S and 18S ribosomal RNA identification, to verify a correct RNA extraction and its integrity.

b) Reverse Transcription (RT)

RT involves the synthesis of cDNA from RNA. To chive that, it was followed supplier's instructions with a reverse transcriptase (Invitrogen©). For this purpose, it was added to an eppendorf 2µL of enzyme Maxima First Strand©, 4µL of reaction mix buffer and 14µL of RNA (in a concentration of 100ng/uL). After 10min at 25ºC, eppendorfs were placed in a hot bath at 50ºC for 30min. Afterwards, eppendorfs were put on ice while the bath temperature rose and once at 85ºC, the samples are placed in the bath, with the aim of degrading the enzyme and ending the reaction. Later, the samples were left to rest on ice for 5 min and centrifuged at 8000G for 40s. Thus, 20uL of cDNA are obtained at approximately 100ng/uL. Those 20uL of cDNA were diluted 1:4 in RNA-Free water to obtain 40uL at 25ng/uL, concentration and quantity necessary to proceed with our genes of interest.

c) qPCR

1 µL of each cDNA was mixed with 9 µL of Master Mix and placed in the qPCR. Master Mix consists of 5µL of SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA), 3µL of nuclease-free water and 1µL of 900:900nM random hexamer primers and oligo-dT (18 mer) primer. Specific primer pairs for genes related to chlorine transport (*kcc1; kcc2*), GABA-A receptor subunits (*gaba-a1α; gaba-a2α*), GABA synthesis (*gad1; gad2*) glutamatergic system (*grin1; grin2a; grin2b; grin2c; grmr*) serotonergic system (5ht-2a; 5ht-2c); neurotrophic factors (*bdnf*) and cholinergic system (*m2r; nicoα7; chat; ache-s*) were used for expression analysis by qPCR. qPCR was conducted in Thermocycler (Applied Biosystems). Samples were duplicated and the relative amount of gene of interest was calculated by the comparative Ct method using the *gadph* promoter specific amplicon as control and its promoter of the intronic region as a negative control (see APPENDIX 2).

PCR conditions were set as in the following Table 1:

Procedure	Master mix (40 cycles)
Denaturation of the DNA	95ºC for 10min
Annealing	60ºC for 1min
Polymerization	95ºC for 15s
Final elongation	60ºC for 1min
For ever	16ºC

Table 1. Conditions for PCR reactions.

All the primers used for qPCR are displayed in Table 2:

Gene	Forward primer	Reverse primer	Source
GAPDH (Intron)	ctgggtggctcaaggaata	cacacgcatcacaaaaaggt	Own design
GAPDH (Exon)	cttcaccaccatggagaag	catggactgtggtcatgag	Own design
NICOα7	tatcaccaccatgaccctga	cagaaaccatgcacaccagt	Chamoun et al.,2016
M2 R	caagacccagtatctccaagtctg	cgacgacccaactagttctacagt	Chamoun et al.,2016
ChAT2	atggccattgacaaccatcttctg	aacaaggctcgctcccacagcttc	Lips et al.,2007
VAChT	gccacatcgttcactctcttg	cggttcatcaagcaacacatc	Lips et al.,2007
AChE-S	gtgagcctgaacctgaagcc	tcctgcttgctatagtggtc	Jameson et al.,2007
GABA-A α1	gcccaataaactcctgcgtatc	attcggctctcacagtcaacct	Fujimura et al.,2005
GABA-A α2	ccaggatgacggaacattgc	ggaaagtcctccaagtgcattg	Fujimura et al.,2005
GAD1	gtgagtgccttcagggagag	cgtcttgcggacatagttga	Own design
GAD2	ctgagaagccagcagagagc	agagtgggcctttctccttc	Own design
KCC1	catgatttcccgctctttg	ccgtacacccgcatgttatt	Own design
KCC2	aggtggaagtcgtggagatg	cgagtgttggctggattctt	Jaenisch etal.,2010
5HT2a	aacggtccatccacagag	aacaggaagaacacgatgc	Kindlundh-Högberg et al.,2006
5HT2c	ttggactgagggacgaaagc	ggatgaagaatgccacgaagg	Kindlundh-Högberg et al.,2006
BDNF	ggtcacagcggcagataa	ccgaacatacgattgggtag	Own design
GRIN1	atggcttctgcatagacc	gttgtttacccgctcctg	Lau et al., 2013
GRIN2a	agttcacctatgacctctacc	gttgatagaccacttcacct	Lau et al. 2013
GRIN2b	aagttcacctatgacctttacc	catgaccacctcaccgat	Lau et al. 2013
GRIN2c	ggcccagcttttgaccttagt	cctgtgaccaccgcaagag	Lau et al. 2013
GRM2	ctatgccacccacagtgatg	gcacagtgcgagcaaagtaatc	Pershina et al. 2018

Table 2. Primers used for RT-qPCR. From left to right, the name of the Gene, forward primer, reverse primer and source. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), GAD1 (Glutamate Decarboxylase 1), GAD2 (Glutamate Decarboxylase 2), GABAA-α1 (Gamma-aminobutyric acid receptor subunit alpha-1), GABAA-α2 (Gamma-aminobutyric acid receptor subunit alpha-2), KCC1 (Potassium/Chloride Transporter Member 4), KCC2 (Potassium-chloride transporter member 5), M2R (Muscarinic 2 receptor), CHAT2 (Choline acetyltransferase 2), VACHT2 (Vesicular acetylcholine transporter), NICOα7 (Nicotinic α receptor), 5-HT2C (Serotonergic receptor 2C), ACHE-S (Acetylcholinesterase isoform S), BDNF (brain-derived neurotrophic factor), 5-HT2A (Serotonergic receptor 2A), GRIN2A (Glutamate receptor ionotropic, NMDA 2A), GRIN2B (Glutamate receptor ionotropic, NMDA 2B), GRIN1(Glutamate receptor ionotropic, NMDA 1) and GRM (Metabotropic glutamate receptor).

Data collection and analysis was performed using StepOne Software v2.2.2 (Applied Biosystem©). Ct average values for every single gene and sample were normalized to its housekeeping gene expression (ΔCt) and then normalized to the averaged ΔCt of the WT, control group (ΔΔCt). This ΔΔCt was then transformed to obtain the fold change (2^ ΔΔCt). Results were calculated following ΔΔCt method in arbitrary units by comparison to a data point from the control samples.

Only those samples that expressed no gDNA contamination, early expression rates for the remaining genes (< Ct30) and no abnormalities in their melting curves (e.g., absence of double/multiple peaks) were accepted for statistical analyses.

3.4. Statistical analyses

For CCT experiment, time in chamber (s), sniffing (s), social efficiency (ratio), SI and SNI variables were equally analysed depending on the specific phase. The factors were GENOTYPE (*fmr1*-KO, WT) and TREATMENT (CPF, control), considered as between-subject variables. These between-subject variables were subsequently used in all the analyses. It was conducted a two-way of variance (ANOVA) for the above-mentioned factors. Pair-wise comparison with SIDAK correction was performed for post-hoc analyses when the main ANOVA reached statistical significance ($p<0.05$).

For genetic experiments, all genes were individually analysed using a two-way ANOVA, with TREATMENT and GENOTYPING as factors. Those outlier samples were eliminated for each group using the tool Outlier Calculator of Graphpad, based on a statistical method, Grubbs' test. In all ANOVA analyses, Sidak correction was applied.

Finally, correlation of CCT variables and gene expression was performed using the software JASP v0.14.0.0. For this purpose, the focus was set on Pearson's r heatmap and correlations.

All ANOVA analyses were performed with SPSS Statistics software v24 (IBM) and represented with Graphad Prism v6. Significance was accepted at the 95% confidence level, *$p<0.05$, **$p<0.01$, ***$p<0.001$). Mean and SEMs are displayed in all the Figures.

4.-RESULTS

4.1. In CCT fmr1-KO adolescent rats show impaired parameters of sociability and CPF exposure can worsen it

For CCT social phase (second phase), CPF exposed *fmr*1-KO rats spent less time in S1 chamber in comparison the other groups (Fig.7A). It was recognized a significant interaction TREATMENT x GENOTYPE [$F(1,45)= 4.080$, $p=0.049$]. Post hoc analysis revealed that CPF exposure primarily in *fmr1*-KO rats, had lower rates of

this behaviour when compared with their control counterparts (p=0,039). Analysing social direct interaction (sniffing), *fmr1*-KO rats were less prone to interact with S1 compared with WT, where exposed rats importantly decreased their direct social interaction rates. Only the condition GENOTYPE reached statistical significance [F(1,45)= 6.378, p= 0.015] (Fig.6B). Next, regarding social efficiency, *fmr1*-KO rats were significantly inefficient, taking more time to get to sniff once they were relatively close to the social target in comparison with WT animals. For this, factor GENOTYPE plays a key role in this behaviour [F(1,45)= 5.748, p= 0.021] (Fig.7C).

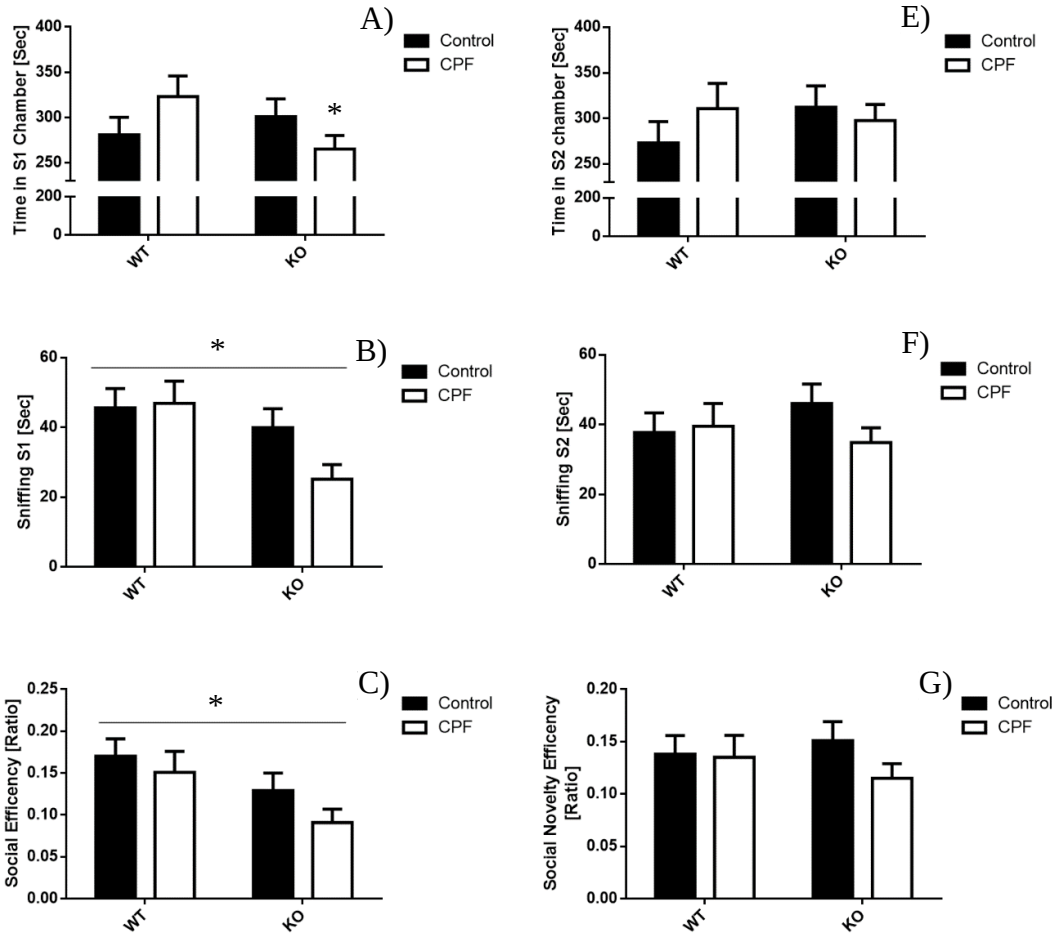

Figure 7. Socialization time, direct interaction (sniffing) and social efficiency obtained for phase 2 and 3 of the 3CC. Figures located in the left hand-side (A,B,C) represent the social phase (phase 2) of 3CC; whilst those disposed at the right (D,E,F) are the results for the phase of reaction to social novelty (phase 3). Right figures (from top to the bottom, figures b, d, and f, respectively) represent the third phase of the testing (reaction to social novelty phase). * indicates significant differences (p<0.05). Data are expressed with means and SEMs.

For reaction to novelty phase (third phase), no significant results were obtained for time spent in S2 chamber (Fig.8D), sniffing S2 (Fig.8E) or social novelty efficiency

(Fig.7F). Nevertheless, *fmr1*-KO rats exhibited higher ratio for SNI, affecting in this behaviour the factor GENOTYPE [F(1,45)= 4.861, p= 0.033] (Fig.8H).

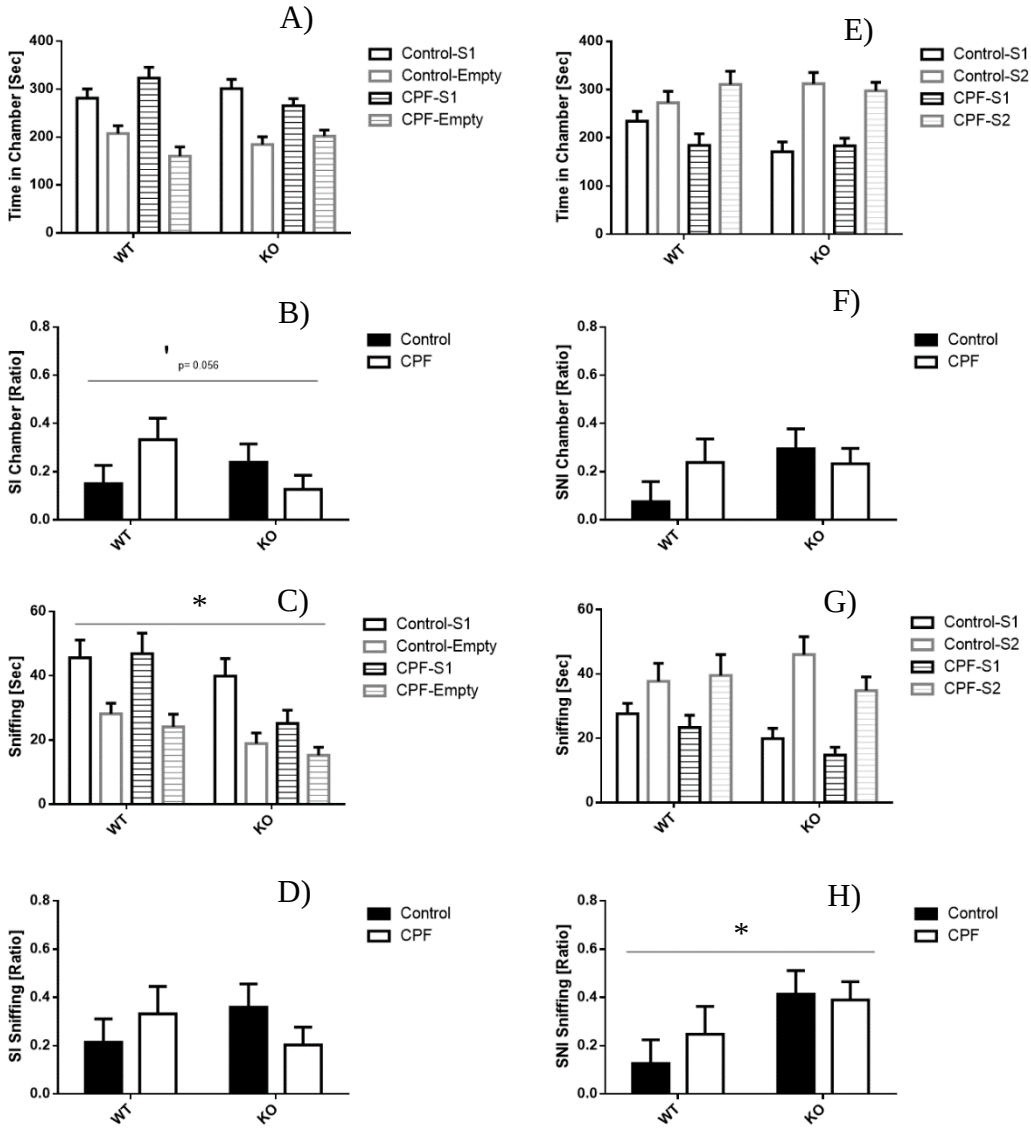

Figure 8. Time spent in each chamber, sniffing of each grid, SI and SNI for both variables obtained for phase 2 and 3 of the 3CC. Figures located in the left hand-side (A,B,C,D) represent the social phase (phase 2) of 3CC; whilst those disposed at the right (E,F,G,H) are the results for the phase of reaction to social novelty (phase 3). * indicates significant differences ($p<0.05$). ‘ indicates a statistical tendency regarding TREATMENT x GENOTYPE interaction. Data are expressed with means and SEMs.

4.2. RT-qPCR revealed CPF-KO hippocampus of adolescent rats show an increase of 5-ht2c and kcc1 expression; whilst there is a rise in grin2c of fmr1-KO

Both CPF exposure in WT rats and non-exposed KO animals had lower relative expression levels of *5-ht2c* in comparison with WT, non-exposed rats, whilst exposed KO animals had similar levels than those observed for the control rats (Fig.9A). A TREATMENT x GENOTYPE interaction was found for this gene

[F(1,40)= 6.537, p=0.014], where exposed KO rats had larger *5-ht2c* relative expression levels in comparison with their non-exposed counterparts (p=0.046).

Other significant value was observed for *kcc1* (K/Cl- Co-transporter type 1) with similar tendency as *5-ht2c*. *Kcc* expression levels increased in those *fmr1*-KO rats exposed to CPF (Fig.9B). This gene expression is affected by the variable TREATMENT x GENOTYPE [F(1,39)=4.414, p=0.042]. Post hoc analysis evidenced that CPF exposition primarily in *fmr1*-KO rats affected this gene expression (p=0.037).

Finally, for *grin2c* (Glutamate Ionotropic Receptor NMDA type subunit 2C) it is observed higher expression for KO rats, independently of the treatment. This is significantly affected by the variable GENOTYPE [F(1,37)= 10.300, p=0.003].

All the other genes analysed with no significant results are available in APPENDIX 3.

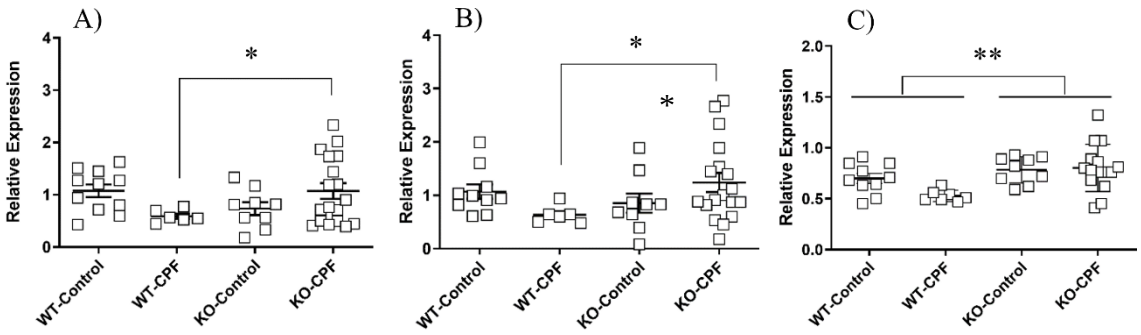

Figure 9. Relative gene expression in the hippocampus of 5-*ht2c*(A), *kcc1* (B) and *grin2c* (C) for each group of rats. * indicates significant differences (p<0.05). ** indicates significant differences (p<0.01). Data are expressed with means and SEMs.

4.3. Gad1, gabaa2 and nicoα7 correlate with CCT sociability variables

After correlating all the genes with the CCT variables, significant values were obtained. For *gad1* and *nicoα7* there was a negative correlation for the variables SOCS1 and SI whilst for *gabaa2* there was negative correlation for the variable OBSF3TIMES2 (Fig.10). All the analysis is available at APPENDIX 4.

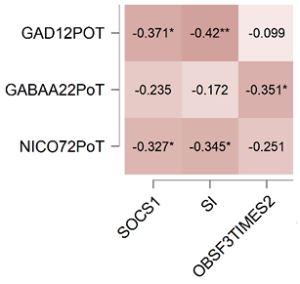

Figure 10. Pearson's r heatmap. Y axis contains the genes *gad1* and *nicoα7* and the X axis the variables SOCS1 (Time in room of Stranger 1 in social phase), SI (social index rooms) and OBSF3TIMES2 (Time sniffing stranger 2 novelty phase) . * indicates p<0.05; ** indicates p<0.01; ***indicates p<0.001.

5.-DISCUSSION

According to the results obtained in this set of experiments, gestational exposure to NChEI doses of CPF during a short period of time in a vulnerable *fmr1*-KO genetic background worsen ASD-like profile in adolescent Sprague Dawley rats regarding social interaction. In CCT phase II, it was observed a decrease in sniffing and social efficiency in *fmr1*-KO rats and a decrease in time spent in chamber with S1 for CPF-KO rats. The only significant result for CCT phase 3 was SNI for *fmr1*-KO, with an interesting and unexpected increase in reaction to social novelty in those KO animals in comparison their WT counterparts. Added to this, we found that both WT and KO genetic backgrounds differently assimilate CPF exposure at a molecular level, where CPF increased the relative expression of *5-ht2c and kcc1* solely in those animals with the genetic vulnerability; whilst in KO rats boosted *grin2c* expression. This is, to the best of our knowledge, the first time that these behavioural and molecular results are obtained regarding CPF exposure and *fmr1*-KO rodent models. Finally, some correlations were found for *gad1, gabaa2* and *nicoα7* and sociability variables of CCT. *Gad1* and *nicoα7* negatively correlate for the variables SOCS1 and SI whilst for *gabaa2* there is negative correlation for the variable OBSF3TIMES2.

In CCT phase 2, it was observed a decrease in sniffing and social efficiency in *fmr1*-KO rats and a decrease in time spent in chamber with S1 for CPF-KO rats. On the whole, the genetic model *fmr1*-KO was more affected in all the results. Other studies have related abnormal social responses in this model with social anxiety, which could be explained as fear of social interaction (Luo et al. 2020; Keil et al., 2019). Moreover, *fmr1*-KO behaviour has been analysed previously reporting impaired attention in the five-choice serial-reaction-time task, increased locomotor activity in some tasks of open-field, altered reverse learning (Zhao & Bhattacharyya, 2018). In a nutshell, *fmr1*-KO shows a conduct very similar to ASD in humans, being considered a well-established ASD model. Moreover, in phase 2 of CCT, there was a reduction in time spent in chamber with S1 for CPF-

KO rats. The results obtained for CPF-KO indicate there must be an interface between these two components. As no other studies have been done previously combining these two factors, it must be discussed with other models in which genetic ASD model is exposed to CPF. Other studies have previously combined genetic and environmental factors with significant social outcomes. Basaure et al. (2019) used APOE4 and APOE3 transgenic mice treated orally with CPF (1-2 mg/kg/dayPND10-15). The authors obtained improved social and novelty interest for APOE4; but contrarily, these behaviours were declined in APOE3 mice for CCT. APOE3 is the isoform commonly found in humans and APOE4 has been related to psychiatric and neurodegenerative problems (Biosca-Brull et al., 2021). This would indicate that, in general terms, this exposure would lead to a decrease in ASD behaviours. In comparison, De Felice et al. (2015; 2016) worked with BTBR mice model and administered CPF via gavage (6mg/kg/day, GD 14-17). They found these animals presented delayed motor maturation, heighten oxidative stress and lipid profile. On the other hand, Mullen et al. (2012) found that transgenic mice with reduced expression of reelin subjected to CPO injection with minipump (6mg/mL/day, GD 13-delivery), exhibited a mitigation of abnormal behaviours. Only these genetic models have been studied in conjunction with CPF exposure. Be that as it may, there is no consensus regarding how CPF affects a genetic ASD-like model.

In CCT phase 3, *fmr1*-KO rats exhibited higher ratio for SNI. This means there is higher curiosity for novelty in KO rats. It could be hypothesized that somehow there is alteration of impulsivity or anxiety pathways, which could increase rat willingness to discover. According to previous articles, *fmr1*-KO mice present social novelty deficit (Jin et al., 2021; Biag et al., 2019), but there is increase hyperactivity and an obsessive-compulsive behaviour which could modify novelty interest (Sidhu, 2014). Furthermore, there was a blurred non-significant tendency related to CPF exposure and interest in novelty; but no significant results related to CPF treatment alone. Anyway, it is interesting discussing bibliography in order to understand how the interaction with *fmr1*-KO can exert this effect. Previous studies observed that OPs gestational exposure (mostly CPF of 1-5mg/kg/day) in mice reduced (Lan et al., 2019,2017) and increased (Venerosi et al., 2010; Aldina et al., 2010) social behaviours in rodent models. The same was observed after postnatal

administration (Berg et al., 2020, Basaure et al., 2019, Venerosi et al., 2008). For example, Venerosi et al. (2010) uncovered that, after the administration of CPF via intraoral gavage (6mg/kg/day, GD 15-18), there is a depression of motor skills and communication in mice pups. Lan et al. (2017; 2019) also observed decrease sociability in CPF treated mice (2.5-5 mg/kg/day, GD 12-15). The outcome here was reduced social conditioned place preference and lowered preference towards an unfamiliar conspecific in the social preference test. In addition, Venerosi et al. (2009; 2010) found a decrease in communication reflected in lower USVs, enhanced anxiety and higher immobility for CPF treated mice (6mg/kg/day, GD 14-17). In general, the social outcome of CPF prenatal exposure means a depression of nervous system and sociability affected negatively. But still is not clear whether this could be considered an ASD-like behaviour.

On the other hand, genetic experiments resulted in three significant differentially expressed genes. Significant results were found in *5-ht2c* and *kcc1* for KO-CPF rats compare with WT; and *grin2c* for *fmr1*-KO. There was up-regulation of *5-ht2c* and *kcc1* in animals exposed to CPF with genetic vulnerability *vs* those not CPF exposed. CPF+KO model was more prone to reach a relative expression closer to the unexposed WT group and in general, higher genetic expression compared with KO or CPF. It could be hypothesized that the mitigation of symptoms could be the outcome of a regulation in genetic expression. CPF can affect protein stabilization and even microtubules and cytoskeleton which could be the basis of the molecular modification (Mullen et al., 2012). 5-*ht2c* codifies a protein that conforms the receptor for serotonin o 5-HT. It binds serotonin, with a critical role in decision-making and social behaviour. Polymorphisms have been linked with ASD, impulsivity, fear, anxiety, drugs addiction, obesity or disrupted communication (Bombaker, 2021; Campbell et al., 2021; Tabbara et al., 2021; Amodeo et al., 2021; Higgins et al., 2020; Stewart et al., 2019; Iritani et al., 2018; Higgins, Zeeb & Fletcher, 2017; Patrick & Ames, 2015). Some studies found a decrease of 5-HT in ASD children (Xi & Wu, 2021), whilst others report the opposite in humans and rats (Qui et al., 2021; O'Reilly et al., 2021; Aldridge et al.,[1] 2005). It may happen that the serotonin lack comes from the known low presence of tryptophan (serotonin precursor) which takes place in ASD pathology (Cetin et al., 2015). 5-HT in addition has the function of regulating gut microbiota

and therefore, if there is a lack of 5-HT this could be the root of ASD dysbiosis (Qui et al., 2021). A study performed by Aldridge et al. [1](2005) in rats exposed to CPF (1mg/kg, GD17-20) observed immediate and persistent effects on down-regulation of 5-HT1A and 5-HT2. Venerosi et al. (2009) found in mice disrupting effects of CPF administration (6 mg/kg, GD 14–18) on serotonergic transmission. They observed up-regulation of synaptic proteins including 5-HT1A and 5-HT2 receptor with a lower 5HT content and a high 5-HT turnover was found after CPF exposure. They also observed lower USV, which is also controlled by circulating serotonin levels. This would mean a decrease in 5-ht tone leads to up-regulation of receptors. Berg et al., (2020) found that the administration of low doses of CPF (1.0 or 3.0mg/kg, PND1-4) decreased rats' USV emissions as well as social exploration. A similar decrease of USV accompanied by increased sniffing was observed by Mullen et al. (2013) in reeler homozygous mice with CPO (6mg/mL, GD13.5). Paradoxically, when both variables (genetic and environmental) are present, there is often a mitigation of altered behaviours, rather than acting as additive factors. This could explain the lower values obtained for social exploration in CCT and the fact that both factors could mitigate symptoms by changes in the neurotransmitter system, which would explain why relative values of CPF+KO almost reach values of WT.

Results for *kcc1* indicate an up-regulation in animals exposed to CPF with genetic vulnerability *vs* those not CPF exposed. *Kcc* encodes the protein SLC12A4 (Solute Carrier Family 12 Member 4) which conform a K+/Cl- symporter (Garneau et al., 2019). It is a critical mediator of synaptic inhibition, a barrier for toxics or drugs and may be a modulator of neuroplasticity and other neurotransmitters as GABA (Pozzi et al., 2020; Lai et al., 2016; Gauvain et al., 2011; Gulyás et al., 2001). GABA activity could be altered by *kcc1* expression; therefore, this could relevant regarding ASD phenotype (Roberts et al., 2021). As *kcc1* has not being studied in depth yet, the other isoform (*kcc2*) with similar functions was searched in bibliography to elucidate alterations in *kcc1* expression. Specific mutations in *kcc2* were associated with ASD and they are suggested to happen by epigenetic modulations (Schulte, Wierenga & Bruining, 2018). As other articles claim, CPF can exert its mechanism through epigenetics, modulating expression of *kcc2* (Guardia-Escote et al., 2020). Moreover, *kcc2* has been observed to decrease in

the presence of a proinflammatory environment and stress, factors commonly found on ASD (Pozzi et al., 2020). In addition, this alteration does not come alone in ASD, usually there is alteration in *nkcc1/kcc2* regulation, being the first one downregulated (Schulte, Wierenga & Bruining, 2018). As was said in the introduction, use of Digoxina with the idea of decreasing inflammation could be a good treatment for these patients. For now, the promising treatment targeting chloride deregulation in ASD is bumetanide. Other approach to increase *kcc2* could be increase of 5-HT2A receptor (Schulte, Wierenga & Bruining, 2018). Though we previously discussed about another serotonin receptor, this connexion of serotonergic system and *kcc2* could explain why both genes have a similar pattern of expression for each condition. In addition, a reduced expression of *kcc2* was observed in post-mortem human brains from FXS and Rett-syndrome (Pozzi et al., 2020). All these data if applicable to *kcc1* would make sense with the lower levels obtained for CPF or KO rats. No results related to the social outcome of the deficient of this gen are available to compare the results of CCT, but as KCC1 regulates neuronal excitation, we could say that alteration of this gen may have a behavioural outcome reflected in movement or social interaction.

Grin2c results indicate that there is overexpression of this gene in all *fmr1*-KO rats, independently of CPF exposure. *Grin* genes codify the protein GluN2A, a subunit of NMDA receptors, which bind to glutamate. They are responsible of maintaining a balance between excitatory and inhibitory activity. NMDA receptors participate in normal brain development, synaptic plasticity, learning and memory, with special interest during gestation. Generally, NMDA subunits alterations can be related to Parkinson's disease, epilepsy, drug addiction, West syndrome, Alzheimer's disease, depression, and schizophrenia (Amin, Moody & Wollmuth, 2021; Uniprot, 2021; Rappaport et al., 2013). Yu et al. (2018) and Tarabeux et al. (2011) found missense mutations in ASD patients for *grin* genes, including *grin2c*, which might increase ASD susceptibility. This gen has not been studied previously neither on *fmr1*-KO or CPF exposure. There is no bibliography to contrast information, but probably as glutamate signalling is altered in ASD, this can be produced by alteration in NMDAR (Colizzi et al., 2021). Moreover, it could explain why sociability is affected.

Finally, the correlations of sociability variables (SOCS1, SI and OBSF3TIMES2) with genetic expression proved the existence of significant interactions with *gad1*, *gabaa2* and *nicoα7*. When trying to understand psychiatric disorders, there is considerable disagreement regarding how genetic influence behaviour and vice versa. It seems like higher expression of *gad1* and/or *nicoα7* could produce an ASD-like behaviour as social interaction decrease (SOCS1 and SI); whilst the interest in novelty (OBSF3TIMES2) increase with higher expression of *gabaa2*. Some studies observed dysregulation of *gad1* in ASD (Sandhu et al., 2014; Zhubi, 2014; Yip, Soghomonian & Blatt, 2007). Gad enzyme was found to be reduced in ASD patients due to an increased binding of the gene MeCP2 to its promoter. This can cause changes in the availability of glutamate and/or GABA and an impaired social interest (Guimarães-Souza et al., 2019). *Nicoα7* has been observed to be reduced in Alzheimer and schizophrenia; whilst overexpressed in ASD (Fonar et al., 2021; Unal et al., 2020; Deutsch et al., 2015). *Nicoα7* is involved in cognitive decline, sensory information processing, attention, executive functions and reward (Fonar et al., 2021; Deutsch & Burket, 2020). Moreover, it is thought to be involved in anxiety, fear and excitability when overexpressed (Perkins et al., 2021). Conceivably, changes in GABAA receptors could affect nicotinic cholinergic input (Deutsch & Burket, 2020). An increase of these parameters could explain why rats are less prone to interact. Even it has been proposed to use nicotinic agonist to improve attention in ASD patients (Deutsch & Burket, 2020). There are no studies for relation of *gabaa2* and social novelty, but through an indirect mechanism (*Erbb4* Deletion), Geng et al. (2017) found there was an increase on GABAA1 receptors and impaired social novelty recognition. All the results point toward the existence of a convoluted network in which all the factors interact with the system and among them. Anyway, it is not clear how to interpret these results, as there were no significant differences between subjects groups.

5. Conclusions and future guidelines

Final conclusions:

1. There is an additive/suppressive effect of both factors for CPF-KO rats, reflected in a decrease of time spent with S1 and increase of *5-ht2c* and *kcc1* expression.

2. Rats *fmr1*-KO are a good ASD model which show a reduction in sniffing, social efficiency and SNI; and a rise in *grin2c* expression.

3. Some sociability parameters (SOCS1, SI and OBSF3TIMES2) correlate with gene expression of *gad1*, *gabaa2 and nicoα7*. For *gad1* and *nicoα7* it was obtained a negative correlation for the variables SOCS1 and SI whilst for *gabaa2* there is negative correlation for the variable OBSF3TIMES2

Be as it may, the bibliography related to changes in genetic expression does not get to definitive conclusions in most of the occasions. Similar issue comes along with behavioural alterations. So still we have to delve further into the basic molecular biology of psychiatric disorders, supported by behavioural testing to get to understand those diseases and try to find a cure for them. Another issue comes when using animal model in neuroscience. Although animal models are so useful for understanding basic molecular mechanisms and fundamental behaviours, it is a fact the dissimilarities with humans (Zhao & Bhattacharyya, 2018). It must be stated that animal models can help us to understand the basics of biology, but human models are needed for studying human neurodevelopmental disorders.

To understand whether neurotransmitters are altered, we could directly perform mass spectrometry analysis to check serotonin or other neurotransmitters levels or by RT-qPCR measuring the enzymes involved in the synthesis pathways (i.e. triptophanhidroksilase). Deeper molecular biology analysis is required to unveil the mechanism beyond this behaviour. As has been explained in the introduction, gut dysbiosis is related to ASD and deregulation of many neurotransmitters. A future proposed project could be analysing the effects of probiotic intake (mostly a mixture of *Bifidobacteria*, *Streptococci* and *Lactobacilli*) as they can partly restores communication, stereotypy and anxiety (Fattorusso et al., 2019; Buffington et al., 2016; Hsiao et al., 2013). On balance, this project offers interesting targets in ASD to which future research could be directed as well as contemplate promising future goals.

7.-BIBLIOGRAPHY

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8.-ANEXES

APPENDIX 1: Physical-chemical characteristics and toxicology of CPF

CPF Physical-chemical characteristics	
Chemical name	CAS: 2921-88-2. *O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate ISO: CHLORPYRIFOS (ing.), CLORPIRIFOS (sp.)
Synonyms	>200 https://pubchem.ncbi.nlm.nih.gov/compound/Chlorpyrifos#section=Depositor-Supplied-Synonyms&fullscreen=true
Brandnames	Lorsban, Dursban, Empire20, Equity and Whitmire PT270
Characteristics	OP pesticide, liquid, flammable, toxic, flash point less than 23 °C
Pictograms	
Appearance	Colourless crystals
Use	broad-spectrum) insecticide, acaricide, miticide and nematicide
Chemical formula	C9H11Cl3NO3PS
Molecular weight	350.57 g/mol
Melting point	41-43.5 ºC
Boiling point	No boiling point at normal pressure; it decompose at 160 °C. This produces toxic and corrosive fumes including hydrogen chloride, phosgene, phosphorus oxides, nitrogen oxides and sulfur oxides. Attacks copper and brass.
Vapor pressure	0.0024 Pa (at 25ºC)
Solubility	1.4 mg/L in water(at 25ºC) 79% w/w in isooctane. 43% w/w in methanol (at 25ºC) Solubility (g/100 g): acetone 650, benzene 790, carbon disulfide 590, carbon tetrachloride 310, chloroform 630, diethyl ether 510, ethanol 63, ethyl acetate >200, isooctane 79, kerosene 60, methanol 45, methylene chloride 400, propylene glycol 4, toluene 150, 1,1,1-trichloroethane 400, triethylene glycol 5, xylene 400. Readily soluble in other organic solvents.
Relative density (g/mL)	1.398 (at 20 ºC)
CPF half-life in surrounding	
Soil	7-140 days
Water	3.5-20 days
Air	4-11h
Plants	2.8-6.7 days

CPF toxicology	
Target organs	Respiratory system, central nervous system, peripheral nervous system, plasma cholinesterase
Exposure symptoms	Wheezing, laryngeal spasms, salivation; bluish lips, skin; miosis, blurred vision; nausea, vomiting, abdominal cramps, diarrhoea
Interactions	Vit C and zinc ease CPF symptoms; whilst ascorbic acid worsen them. CPF increases ethanol sensitivity,
Acute Reference Dose (RfD)	5×10^{-3} mg/kg/day
Chronic RfD	3×10^{-4} mg/kg/day
Allowed tolerance for food commodities	0.1-8ppm. Being the highest for Corn, sweet, stover, forage, beet, tops, peppermint and oil
EPA tolerance level in edibles	0.1-1ppm
NOAEL dosis	0.1 mg/kg/day for short- and intermediate-term inhalation shows no observed effect in humans
CPF detection (North Carolina)	
Home	19- 154.2 ng/m^3
Outdoors air	33 ng/m^3
Edible fruit	<0.03ppm
Toxicology LD_{50} rats	
Oral	95 to 270 mg/ kg
Dermal	>2,000 mg/kg
Inhalation	>0.2 mg/L
CPF half-life	
Rats (dose 50 mgCPF/kg oral)	In tissue <20h, whilst in fat 62h
Rats (0.2 mmolCPF/kg, intraperititoneally injected)	Blood 8.1-24.6h
Human (1mg CPF, oral)	Urine 15.5-27h
Human (28.59mg CPF, dermal)	Urine 30h

(National Center for Biotechnology Information, 2021; Smegal, 2000)

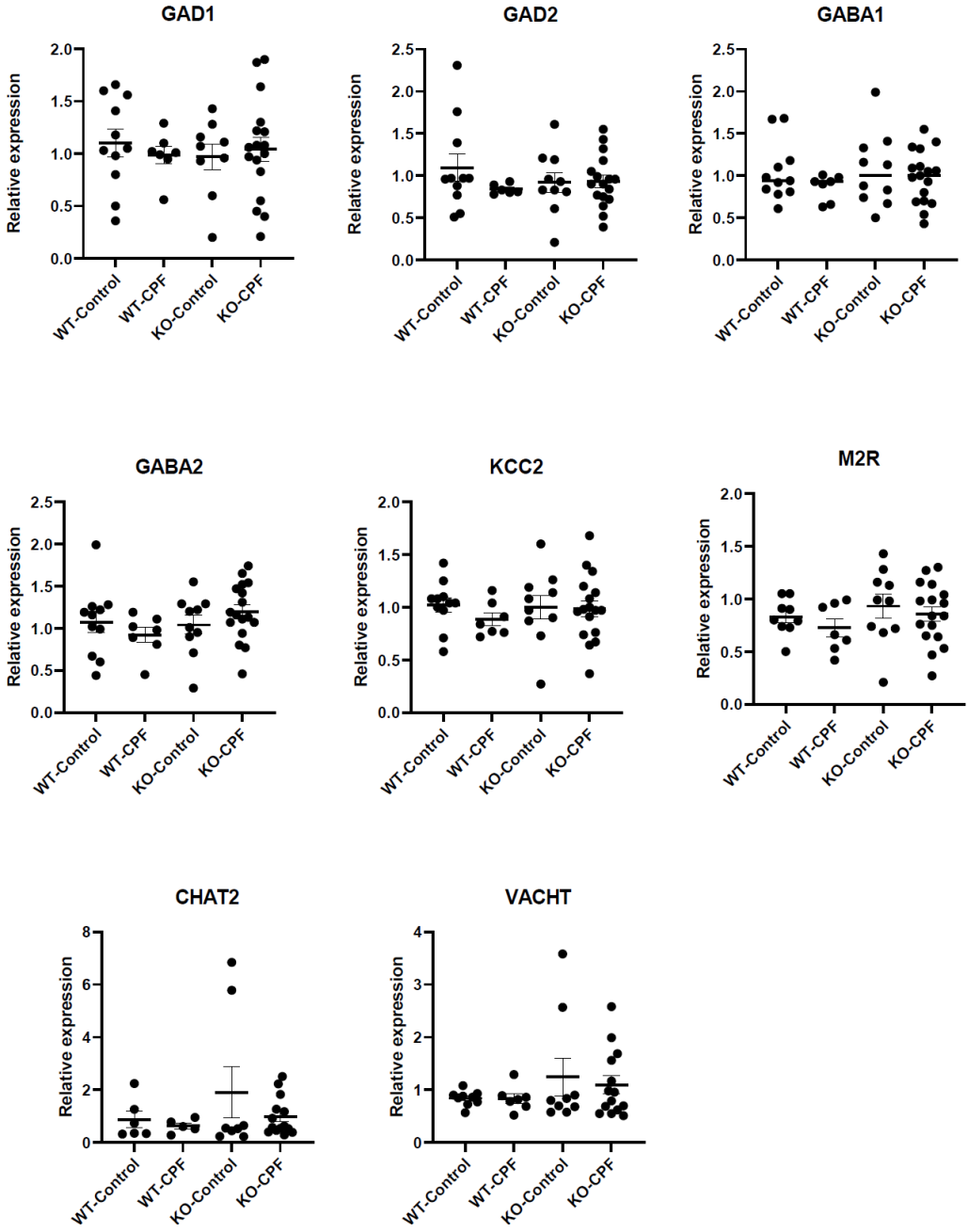
APPENDIX 2: GENES OF STUDY RT-qPCR

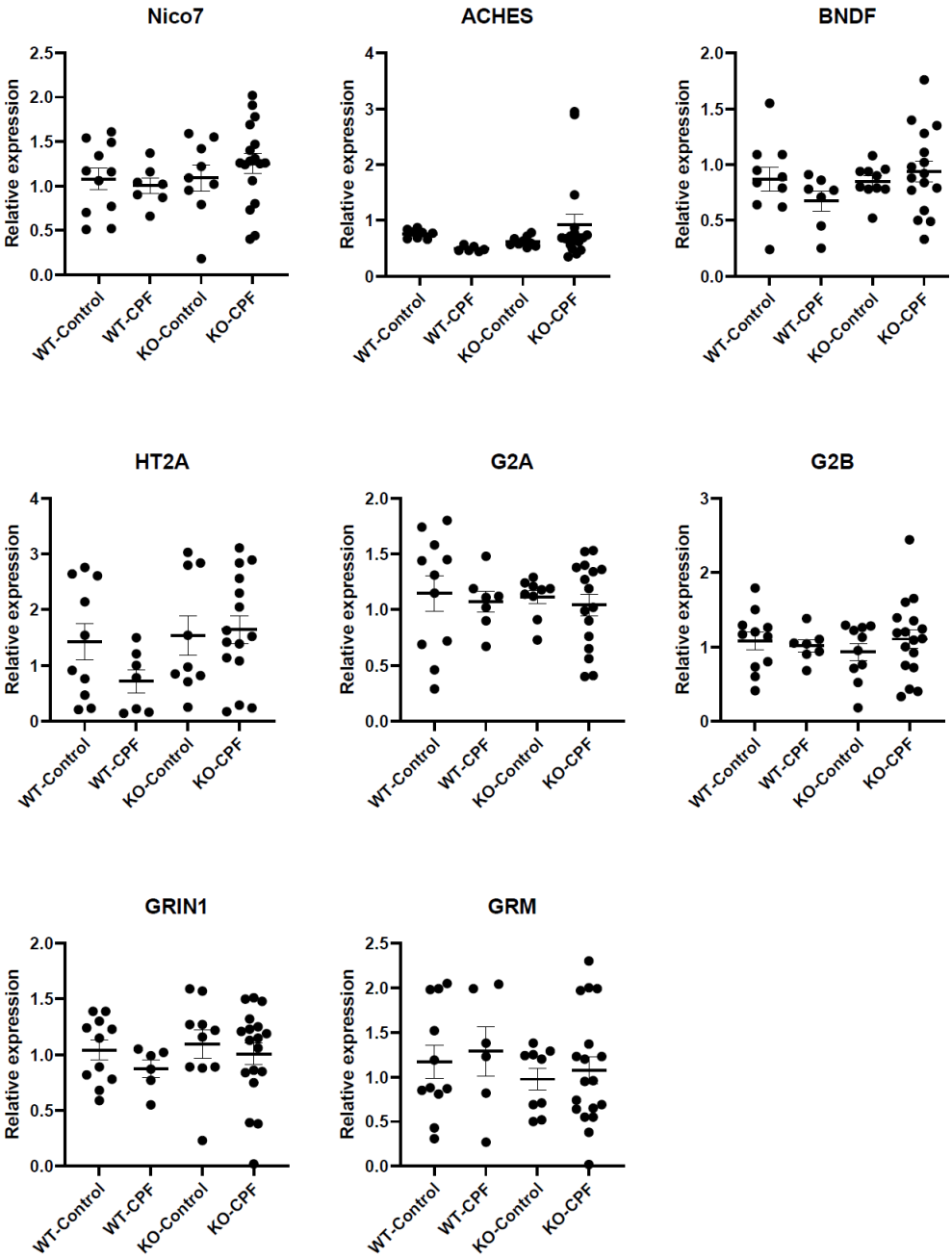
Type	Gen	Description
Positive control	HK, GADPH	Exonic region of the GADPH gene, that is expressed ubiquitously. After doing reversal transcription, the cDNA obtained will contain only exonic regions as we part from RNA (Ct<30).
Negative control	Geno	This is a primer for the intronic region of GADPH gene. cDNA retrotranscribed differs from gDNA in the absence of intronic regions. Therefore, if there is no gDNA contamination (Ct>30).
Chlorine transport	*kcc1*	*Kcc* encodes a neuron-specific chloride-potassium symporter, responsible of establishing the chloride ion gradient in neurons through the maintenance of low intracellular chloride concentrations (Garneau et al., 2019). It is a critical mediator of synaptic inhibition, cellular protection against excitotoxicity and may also act as a modulator of neuroplasticity (Pozzi et al., 2020; Gauvain et al., 2011; Gulyás et al., 2001). It determines the postsynaptic effect of GABAergic transmission. There are 4 isoforms of this transporter. *Kcc1* synthetizes SLC12A4 (Solute Carrier Family 12 Member 4), the most abundant isoform and is a ubiquitous protein located in high amounts in cell membrane and lysosomes. This protein has been related to erythropoiesis, sickle cell formation, cancer growth, bone turnover, fish-eye disease and lecithin: cholesterol acyltransferase deficiency (Garneau et al., 2019). Apart from K+/Cl-, it can transport sugars, bile salts or metal ions. It has been related to erythropoiesis, sickle cell formation, cancer growth, and bone turnover, but no studies has been done on ASD (Garneau et al., 2019). All the protein information available at https://www.uniprot.org/uniprot/Q9UP95 *Kcc2* synthetizes SLC12A5 (Solute Carrier Family 12 Member 5) it is involved in the regulation of dendritic spine formation and maturation. Some studies demonstrated a decrease of KCC2 in Rett syndrome patients (Schulte, Wierenga & Bruining, 2018) and variants of this gen are presented in epilepsy. All the protein information available at https://www.uniprot.org/uniprot/Q9H2X9
	kcc2	
GABA-A receptor subunits	*gaba-a1α*	This genes codifies proteins that belongs to the GABA receptor. There are two kind of GABA receptors: $GABA_A$ or ionotropic and $GABA_B$ or metabotropic (Cetin et al., 2015) It seems that activation of GABA-A receptors in immature neurons produce depolarization instead of hyperpolarization, due to elevated intracellular concentration of chloride in immature neurons that keeps a depolarized reversal potential of GABA-A receptor mediated responses (Leinekugel, 2003). GABA is altered in ASD patients (Coghlan et al., 2012). GABA-A receptor system appears to have functional roles in neurodevelopment. For example, α1 subunit is involved in plasticity in the developing visual cortex. Moreover, α2 is a target of some drugs like diazepam. It is suggested that GABA system could be downregulated in ASD; as there is GABA dysfunction in anxiety or epilepsy disorder, also symptoms of ASD (Coghlan et al., 2012). Moreover, $GABA_A$ and $GABA_B$ receptor subunit in various brain regions significantly decrease. Reduced production or signalling of GABA cause hyperexcitability state and leads to cognitive dysfunction (Cetin et al., 2015). All the protein information available at https://www.uniprot.org/uniprot/M9TKJ0
	gaba-a2α	
Glutamatergic system	*grin1*	*Grin* genes codify the protein GluN2A, a subunit of NMDA receptors, which are glutamate-gated ion channels permeable to calcium, potassium, and sodium. NMDA receptors participate in normal brain development, synaptic plasticity, learning and memory, with special interest during gestation. Its presence includes brain regions of speech and language. Generally, NMDA subunits alterations can induce Parkinson's disease, Alzheimer's disease, depression, and schizophrenia. Deleterious missense and nonsense variants in *GRIN1*, *GRIN2A-D*, and *GRIN3A-B* cause encephalopathies that are sometimes first diagnosed as intellectual disability, global developmental delay, epilepsy, autism, and/or schizophrenia (Mielnik et al., 2020). Specially, *grin1* play a key role in the plasticity of synapses, which is believed to underlie memory and learning. Decreased social interaction in GRIN1-knockout mice was observed in two separate studies (Rosi et al., 2017). All the protein information available at https://www.uniprot.org/uniprot/Q05586 *Grin2a* gene mutation have been found in epilepsy-aphasia spectrum and is involved in opioid addiction.
	grin2a	
	grin2b	

Glutamatergic system	*grin2b*	Mutations in *grin2b* can produce affectations of nervous system as West syndrome and Autosomal dominant non syndromic intellectual disability. All the protein information available at https://www.uniprot.org/uniprot/Q12879
	grin2c	In addition, diseases linked with *grin2c* alteration include arthogryposis and schizophrenia (Uniprot, 2021; Rappaport et al., 2013). Grin2c is located chiefly postsynaptic as a subunit of NMDA receptor and is responsible in pyramidal neurons of maintaining a balance between excitatory and inhibitory activity (Uniprot, 2021). All the protein information available at https://www.uniprot.org/uniprot/Q14957
	grm	The metabotropic glutamate receptors are members of the group C family of G-protein-coupled receptors, or GPCRs. Metabotropic receptors (mGluR) are coupled with G protein, subdivided in 3 groups. On the other hand, ionotropic receptors are coupled with ion-channel and ssubdivided in NMDA, AMPA and kainite receptors (Cetin et al., 2015). They bind glutamate and modulate synaptic transmission and neuronal excitability. Glutamate is the main excitatory neurotransmitter of CNS, synthesized from glutamine via glutaminase enzyme. It is involved in learning, memory, anxiety, and the perception of pain (Niswender & Con., 2010). In ASD, it is not clear if there is a hyper or hypoglutamatergic alteration; but it is clear that there is dysfunction in the glutamatergic system (Cetin et al., 2015).A defective network of metabotropic glutamate (GRM) receptor signalling was found in both ADHD and schizophrenia, two neuropsychiatric disorders that are highly coincident with the ASD (Lee et al., 2015). Moreover, it can trigger when deregulated Alzheimer's disease, Parkinson's disease, anxiety, depression (Niswender & Con., 2010). All the protein information available at https://www.uniprot.org/uniprot/P23385
Serotonergic system	*5-ht2a*	Serotonin receptors can be classified in 7 families of G-proteins. From the biological point of view, serotonin neurotransmitter system has critical role in the regulation of crucial steps of neuronal development such as cell proliferation, differentiation, migration, apoptosis synaptogenesis, neuronal and glial development (Cetin et al., 2015). Serotonin receptors modulate the release of many neurotransmitters including glutamate, GABA, dopamine, epinephrine / norepinephrine, and acetylcholine, as well as many hormones, including oxytocin, prolactin, vasopressin, cortisol, corticotropin, and substance. Moreover, they regulate other neurobiological processes affected in ASD. 5-HT2A is mainly excitatory, although may also have an inhibitory on certain areas such as the visual cortex and the orbitofrontal cortex. It has been reported to be related to schizophrenia (Latorre et al., 2019; Qesseveur et al., 2016). All the protein information available at https://www.uniprot.org/uniprot/Q9VN38
	5-ht2c	The 5-HT2C receptor is a significant modulator of the hypothalamic–pituitary–adrenal axis. This activation can contribute to depressive and anxiety symptoms seen in many psychopathological conditions as ASD. It has been described a reduction in the levels of serotonin in the volume of the dorsal raphe nucleus (Nakai et al., 2017). All the protein information available at https://www.uniprot.org/uniprot/P28335 In ASD, it is not clear if there is hyper or hyposerotonergic state; but generally, serotonin is high in blood whilst lower in brain. Low tryptophan usability in ASD patients might be linked with the serotonergic dysfunction (Cetin et al., 2015),
Neurotrophic factors	*bdnf*	Brain-derived neurotrophic factor promotes during development the survival and differentiation of selected neuronal populations of the peripheral and central nervous systems, participates in axonal and dendritic growth and modulates neuroplasticity. The versatility of BDNF is emphasized by its contribution to a range of adaptive neuronal responses including long-term potentiation (LTP), long-term depression (LTD), certain forms of short-term synaptic plasticity, as well as homeostatic regulation of intrinsic neuronal excitability. All the protein information available at https://www.uniprot.org/uniprot/P21237 Some studies have found lack of association with ASD (Lyall et al., 2021) whilst others found some correlations (Robinson-Agramonte et al., 2021).
Cholinergic system	*m2r*	Muscarinic acetylcholine receptor mediate acetylcholine-induced neurotransmission and five subtypes have been identified. It is involved in Alzheimer disease (Jiang et al., 2014). It is involved in the development of cognitive impairments observed in neuropsychiatric disorders such as schizophrenia (Gould et al., 2015). All the protein information available at https://www.uniprot.org/uniprot/Q09388

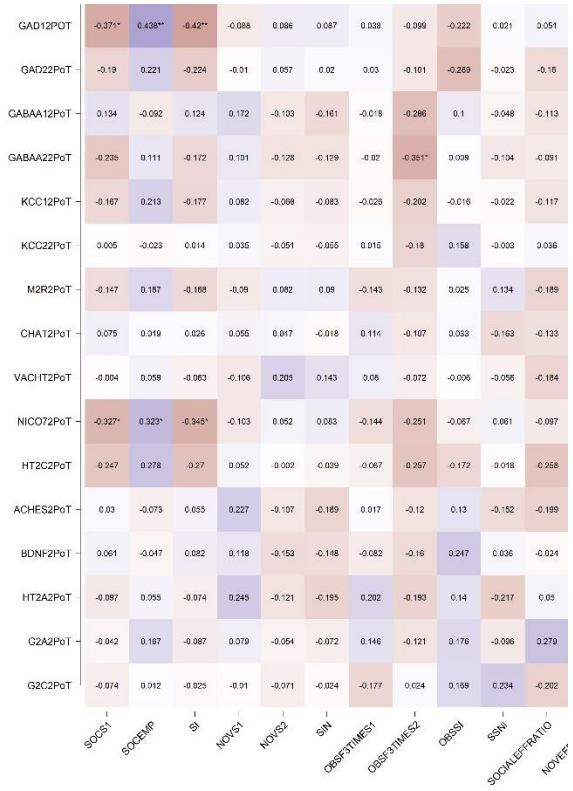
Cholinergic system	*nicoα7*	Nicotinic receptors are ion channels capable of responding to the chemical mediator acetylcholine or nicotine. The receptor has a pentameric structure. it allows the entry of Na + into the cell or the exit of K +. There are also some configurations that allow the passage of Ca +. α7-subunit containing nicotinic acetylcholine receptor (nico α7) is implicated in Alzheimer's disease, causing a cascade that results in cytotoxicity and deposition of amyloid plaques (Deutsch et al., 2015). As acetylcholine is altered in ASD, it should be expected to observe changes also in this gene expression.
	chat	This gene encodes an enzyme choline acetyltransferase, which catalyzes the biosynthesis of the neurotransmitter acetylcholine. Polymorphisms in this gene have been associated with Alzheimer's disease and mild cognitive impairment; but also congenital myasthenic syndrome. Multiple transcript variants encoding different isoforms have been found for this gene, and some of these variants have been shown to encode more than one isoform (Suzuki et al., 2011). As acetylcholine is altered in ASD, it should be expected to observe changes also in this gene expression. All the protein information available at https://www.uniprot.org/uniprot/P28329
	ache-s	It encodes a hydrolase that hydrolyzes choline esters. Elevation of Ach levels led to a significant improvement in cognitive rigidity, social preference and social interactions, all core symptoms in ASD patients. Systemic treatment with the AChEI (S) [acetylcholinesterase suppressor] Donepezil rescued the autistic-like phenotype in independent and complementary behavioral tests (Karvat & Kimchi, 2013)

APPENDIX 3: Relative expression of non-significant genes




Nico7
ACHES
BNDF
HT2A
G2A
G2B
GRIN1
GRM
Relative expression
WT-Control
WT-CPF
KO-Control
KO-CPF

APPENDIX 4: Person's r test. Comparison gene expression VS CCT variables



SOCS1 (Time in room of Stranger 1 in social phase), SOCEMP (time in empty box room in social phase), SI (social index rooms), NOVS1 (time in room in box with stranger 1 novelty phase), NOVS2 (Time in room box with stranger 2 novelty phase), SIN (Novelty index rooms), OBSF2TIMES1 (Time sniffing stranger 1 in social phase), OBSF2TIMEEMPTY (Time sniffing empty box social phase), OBSF3TIMES1 (Time sniffing stranger 1 phase novelty), OBSF3TIMES2 (Time sniffing stranger 2 novelty phase), OBSSI (Social index social phase for sniffing), OBSSNI (Novelty index novelty phase for sniffing), SOCIALEFFRATIO (Social efficiency in social phase), NOVEFFRATIO (Novelty efficiency novelty phase).