para-Chlorophenylalanine induces aggressive behavior by serotonin depletion in male rats and increases tryptophan hydroxylase 2 and GABAA α1 mRNA expression in the olfactory bulb.

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Abstract

Decreased serotonin (5-HT) has long been linked to increased aggressive behavior. Tryptophan hydroxylase (TPH) is an enzyme involved in 5-HT synthesis and para-chlorophenylalanine (pCPA) inhibits its activity. TPH2 mRNA expression presence has been mainly described in the raphe complex rodent's brain. 5-HT-producing neurons in the raphe project their axons to olfactory bulb, considered to be a relevant structure in rodents for establishing social interactions, including aggressive behavior. However, the relationship between olfactory bulb and aggression in a pCPA 5-HT depletion model has not been studied. Moreover, receptor subunit GABA α1 has been found in the olfactory bulb and 5-HT depletion could affect GABAA receptor expression in different brain areas. Thus, our aim was to evaluate aggressive behavior, serotonergic activity, the TPH2 and GABAA α1 mRNA expression, in the olfactory bulb, after a single pCPA (300mg/kg) or vehicle i.p. administration in male rats. Aggression was tested using a resident intruder test. Sequentially, the olfactory bulb was obtained, and neurochemical and molecular techniques were used to measure 5-HT, 5-HIAA, TPH2, and GABAA α1 mRNA expression respectively. pCPA administration increased aggressive behavior parameters, without affecting locomotion, nonsocial or social interaction. 5-HT levels were decreased after pCPA administration, as well as its turnover rate, although there were no significant changes in 5-HIAA. TPH2 mRNA expression was increased. GABAA α1 mRNA expression was increased in the olfactory bulb. Our results apport evidence to the serotonergic deficiency hypothesis of aggression and highlight olfactory bulb role as an important structure for understanding aggressive behavior neurobiological complexity.

Introduction

Aggressiveness is considered a social behavior since it requires at least two subjects, and it can be either defensive or offensive. Among primates, humans and rodents there are similarities in aggressive neurobiology (Takahashi et al., 2011; Niederkofler et al., 2016). The aggressive spectrum displayed depends on the specie, the sex, the stimuli, and the contextual contingency (Niederkofler et al., 2016; Takahashi et al., 2011; Takahashi & Miczek, 2014). According to the qualitative and quantitative features aggressiveness in rodents, could be considered as adaptive or maladaptive (Muroy et al., 2016). In their natural environment, rodents are strongly aggressive to defend their food resources, reproductive possibilities and territory (Takahashi et al., 2012). The resident vs. intruder test (RVI) is the most validated paradigm to measure this kind of interaction (Takahashi & Miczek, 2014).

Serotonin (5-HT) plays an important role in social behavior. Among others, modulates several behaviors,

such as copula, postnatal care, adolescent social playing, maternal and territorial aggressiveness (Berger et al., 2009; Buhot et al., 2000; Mittal et al., 2017; Strüder & Weicker, 2001; Takahashi et al., 2012). Many systems are involved in aggressive behavior (Chamero et al., 2011; Carrillo et al., 2009; Duke at al., 2013) Although, serotonergic deficiency hypothesis establish an inverse relationship between the serotonergic system activity and aggressive behavior (Cervantes & Delville, 2009; Kravitz & Huber, 2003; Miczek et al., 2004; Mongillo et al., 2014; Takahashi et al., 2011; Niederkofler et al., 2016; van Erp & Miczek; 2000). In rodent models, there are several ways to generate aggressiveness by decreasing 5-HT: neurotoxicity, olfactory bulbectomy, social isolation, and pharmacological depletion (Hritcu et al., 2007; Jéquier et al., 1967; Koe & Weissman, 1966; Vergnes et al., 1988). Para-chlorophenylalanine (pCPA) is an irreversible TPH enzyme inhibitor. An intraperitoneal pCPA administration causes an acute decrease within the central serotonergic system (Jáquier, 1967; Hritcu et al., 2007) and it has been used to generate aggression models (Jáquier, 1967; Keleta et al., 2007; Kubala et al., 2008).

5-HT producing neurons in the raphe complex project their axons to the cortex, amygdala, hippocampus, basal ganglia, thalamus, hypothalamus, and olfactory bulb (OB) (Lesch & Waider, 2012; Mazerolle et al., 2016). OB is an important serotonergic innervated structure (Huang et al., 2017; McLean & Shipley, 1987; Steinfeld et al., 2015) and the main sensory information used by rodents to start social interactions, such as aggressive behavior, is olfaction (Guillot & Chapouthier, 1996). Moreover, Lucki (1998) showed that 5-HT synthesis inhibition in OB rats caused increased aggressiveness, whereas enhancement transmission suppressed aggressive behavior. First sensory center that processes odor information is the OB, a structure that has been considered when studying territorial aggression (Bester-Meredith et al., 2022). Nonetheless, the relationship between OB and aggression in a pCPA 5-HT depletion model has not been studied.

5-HT is synthesized through the tryptophan hydroxylase (TPH) enzyme (Khan & Thomas, 2004). TPH gene has two isoforms (Walther et al., 2003), while the TPH1 isoform is mostly expressed outside the blood-brain barrier, the TPH2 isoform is expressed in the cerebral tissue and its mRNA expression has so far been mostly described in raphe complex neurons (Malek et al., 2005; Patel et al., 2004; Pelosi et al., 2015; Walther et al., 2003). A human brain post-mortem study, showed TPH2 expression in the cortex, thalamus, hypothalamus, hippocampus, amygdala, cerebellum, and raphe nuclei (Zill et al., 2007). A study using catfishes (Raghuveer et al.; 2011) reported TPH2 mRNA expression in OB. Furthermore, Patel et at., (2004) showed that TPH2 mRNA expression in rat brain revealed a very weak signal in the OB.

Principal neurons and local interneurons in OB have GABAA receptors with different subunit components (Panzanelli, 2005). Particularly, the presence of GABAA receptor subunit α1 mRNA in the OB has been described (Zhang et al., 1991) and is the most common GABAA α subunit in adult rodent neurons (Bosman et al., 2002). In addition, it has been reported that 5-HT depletion could affect GABAA receptor expression in different brain areas (Si et al., 2022; Wang et al., 2020).

Our aim was to evaluate aggressive behavior, serotonergic activity in the OB, TPH2, and GABAA α1 mRNA expression in the OB, after a single pCPA i.p administration in male rats.

Methods and Materials

Subjects and housing

Animals were housed in a temperature-controlled animal room (22 +/- 2 °C) on a 12 h light/dark cycle (light on from 07:00 to 19:00), with artificial light (60-70 lumens). Food and water were available ad libitum.

RVI test requires a resident subject and an intruder. Residents were Sprague-Dawley male rats of 60 days and 350 g (average weight). Intruders were Sprague-Dawley male rats of 50 days and 280 g (average weight). Resident subjects were divided into 2 groups (n=10). Experimental subjects were injected with pCPA and returned to their home cage for 6 days; control subjects were injected with a vehicle and returned to their home cage, at the same time. After the RVI test residents were euthanized and OB were removed for posterior analysis (Koolhaas et al., 2013). Intruder subjects remained housed in groups of 3 until the test and they were euthanized after it.

Experimental Design

Between 15:00h and 18:00h animals from the experimental groups received a single pCPA (Sigma Aldrich, 2015) i.p. injection (dose 300 mg/kg), solved in sterile saline. After pCPA administration residents were housed individually in their homecages until RVI test day, which was performed six days later. Then animals were euthanised and OB were obtained for neurochemical and genetic analysis.

Behavioral Testing

To assess aggressive behaviors, RVI was used. RVI test was applied according to Koolhaas et al. (2013) with modifications. Briefly, the RVI test consists of the interaction of two subjects: the experimental animal, named the resident, and the interaction animal, named the intruder. RVI test was performed 6 days after pCPA administration, between 15:00h and 18:00h (Corthell et al., 2013). In order to verify that residents were heavier than intruders, the animals were weight before the test. The test was carried out in a wood box with the wood chips from the resident home cage. The box was cleaned after each test, with ethanol (10%) (Casas et al., 2011). All the tests were recorded with the Everio G-series GZ-MG330 JVC camera, from above the field. Behavioral analysis was hand scored watching the videos in blind.

RVI test total time was 900 seconds. RVI test total time was divided into two phases, the adaptation phase, and the interaction phase. The first 300 seconds were considered the resident adaptation phase to the environment. 600 seconds remaining were considered the interaction phase. At the beginning of the interaction phases, an intruder was placed on the opposite side of the resident at the end of the adaptation phase and the interaction behaviors were measured. We evaluated the animal locomotor activity as the travel distance during the adaptation phase. In the interaction phase, we evaluated four main groups of behaviors: 1-nonsocial activity, as time grooming, sniffing, and sitting; 2-social activity as the time of heterogrooming and heterosniffing; 3-aggressive behavior, as an event of the chase, moving towards, upright posture and false mount, bite, clinch, clinch attack, lateral threat and keep down were evaluated in the resident during interaction phase; and 4-aggressive latency, as the time preceding the first aggressive behavior. The criteria to discontinue resident interactions with the intruders were bite to delicate body parts e.g. belly, throat and paws.

RNA extraction and Real Time PCR analysis

Total RNA from all left and right OB tissue was extracted using the TRIzol reagent, according to the manufacturer specifications (Invitrogen-Life Technologies, Buenos Aires, Argentina.). mRNA integrity samples were confirmed by 1% agarose gel electrophoresis and staining with Sybr Gold (Invitrogen-Life Technologies, Buenos Aires, Argentina). 10 µg

The mRNA levels of TPH2 and GABAA α1 were estimated by RT real-time PCR with a Corbett Rotor-Gene 6000 Real-Time Thermocycler (Corbett Research Pty Ltd (Sydney, Australia) using rat-specific primers and reaction conditions described in Table 1. The PCR reactions were performed using a Corbett Rotor-Gene 6000 Real-Time Thermocycler using Eva-GreenTM (Biotium, Hayward, CA) in a final volume of 20 µL. The reaction mixture consisted of 2 µL of 10×PCR Buffer, 1 µL of 50 mM MgCl2, 0.4 µL of 10 mM dNTP Mix (Invitrogen), 1 µL of 20× Eva Green, 0.25 µL of 5 U/µL Taq DNA Polymerase (Invitrogen) 0.1 µL of each 2.5 mM primer (forward and reverse primers) and 10 µL of diluted cDNA. The PCR reactions were performed under the conditions described in Table 1. Melt curve analysis was used to check that a single specific amplified product was generated. Real-time quantification was monitored by measuring the increase in fluorescence caused by the binding of EvaGreen dye to double-strand DNA at the end of each amplification cycle. According to the manufacturer protocol, the relative expression was determined using the Comparative Quantitation method of normalized samples about the expression of a calibrator sample (Pfaffl, 2001). Each PCR run included a no-template control and a sample without reverse transcriptase. All measurements were performed in duplicate. The reaction conditions and quantities of cDNA added were calibrated such that the assay response was linear concerning the amount of input cDNA for each pair of primers. RNA samples were assayed for DNA contamination by performing the different PCR reactions without prior reverse transcription. Relative levels of mRNA were normalized to the S16 reference gene.

The real-time PCR products were analyzed on 2% agarose gels containing 0.5 mg/mL ethidium bromide and a unique band of the approximately correct molecular weight corresponded with a unique peak in melt curve analysis.

The Real-Time PCR reactions were carried out for 40 cycles with an initial step of 5 min at 95 ºC followed by a three-step scheme: 30 s at 95 ºC, 30 s at the annealing temperature shown above for each primer pair, and a final step at 72 ºC for 30 s. Primer's design was done with Beacon Designer 7.9 software.

5-HT and 5-HIAA content determination by HPLC in the olfactory bulb

All OB tissue homogenization was performed according to Chi et al., 1999. Briefly, the tissue was collected in 400 µl of 0.2 N perchloric acid and then homogenized in a glass-glass homogenizer. The homogenate was centrifuged at 12000×g for 15min at 4°C (Hermle LaborTechnik GmbH, model Z233MK-2) and the supernatant was injected into a high-performance liquid chromatography (HPLC) instrument coupled to electrochemical detection, to measure 5-HT, 5-HIAA. The pellet was resuspended in 1N NaOH for protein quantification by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Richmond, CA, USA) using bovine serum albumin as standard. The contents of 5-HT and 5-HIAA were expressed as picograms per milligram of total protein. 10 µl of each supernatant were injected into the HPLC system with the following setting: A isocratic pump, (model PU-2080 Plus, Jasco Co. Ltd., Tokyo, Japan), a UniJet microbore column (MF-8912, BAS, West Lafayette, IN, USA), and an amperometric detector (set at 650 mV, 0.5 nA; model LC-4C, BAS, West Lafayette, IN, USA). The mobile phase, containing 0.05 M NaH2PO4, 1.0 mM 1-octane sulfonic acid, 0.27 mM EDTA, 1.0% (v/v) tetrahydrofuran, and 4.0% (v/v) acetonitrile (CH3CN) (pH adjusted to 2.6) was pumped at a flow rate of 100µl/min. The level of neurotransmitters and metabolites was assessed by comparing the respective peak area and elution time of the sample with a reference standard. The quantification was performed using a calibration curve for each neurotransmitter (Program ChromPass, Jasco Co. Ltd., Tokyo, Japan). Under these experimental conditions, retention times were 33.3 for 5-HT and 25.6 for 5-HIAA. Standards, EDTA, and 1-octane sulfonic acid were purchased from Sigma-Aldrich, Inc. (St Louis, MO, USA), and all other reagents were of analytical grade.

Statistical Analysis

All data were analyzed by t-test. Shapiro-Wilks test was previously performed on each group to determine normal distribution. The significance level was set at $p < 0.05$ for all statistical tests. Data were expressed as means ± S.E.M of 10 rats per experimental group. All data were analyzed using Statistica's software application (StatSoft, Krakow, Poland).

Results

Behavioral Assays

The pCPA effect in behavioral evaluation. We found that pCPA single administration, did not affect travel distance ($p= 0.3296$; $t= 1.004$; $df=18$) (Table 2) when compared to the control group. Moreover, pCPA did not affect nonsocial activity ($p= 0.1746$; $t= 1.417$; $df= 18$) and social activity ($p= 0.1957$; $t= 1.347$; $df= 18$) (Table 2). However, pCPA induced a significant increase in aggressive behavior with respect to the control group ($p < 0.001$) (Figure 1-A); and significantly decreased aggressive latency ($p < 0.0001$) (Figure 1-B).

5-HT and 5-HIAA content determination by HPLC in the olfactory bulb

The pCPA effect in neurochemical assays in the OB. We found that pCPA single administration, decreased significantly 5-HT concentration ($p < 0.01$) and serotonergic turnover (5-HIAA/5-HT) ($p < 0.05$) in OB; 5-HIAA concentration was not affected ($p= 0.7982$; $t= 0.2591$, $df= 18$) (Table 3).

Real Time PCR analysis

The pCPA effect on TPH2 and GABAAα mRNA expression in the OB. We found that pCPA single administration significantly increased ($p<0.05$) TPH2 mRNA expression in OB (Figure 2-A). Also, it significantly increased GABAAα1 mRNA expression ($p<0.05$) in OB (Figure 2-B).

Discussion

The initiation, maintenance, and termination of aggressive behavior activates complex neurobiological circuits, among which the serotonergic system is strongly involved (Niederkofler et al., 2016; Takahashi & Miczek 2014). In the current study, using a RVI paradigm, we studied aggression behavior induced by pCPA and the relation with serotonergic activity, TPH2 and GABAAα1 mRNA expression in the OB.

It is well accepted that when evaluating social interaction, it becomes necessary to discard locomotor activity alterations. (Miczek et al., 2013; Takahashi & Miczek 2014). A locomotor activity alteration in the resident could prevent the correct measurement of aggressive behavior, in particular the aggression latency parameter. Interestingly, 5,7-dihydroxytryptamine lesions, used to deplete central 5-HT did not affect locomotor activity (Vergner et al., 1988; Hole et al., 1977). Nonetheless, previous works utilizing pCPA 1000 mg/kg doses found a substantial decrease in locomotor activity (Dringenberg et al., 1995; Matte & Tornow, 1978). Significantly reduced locomotor activity was also seen following chronic treatment with lower dosage of pCPA (100 mg/kg) (Keleta et al., 2007 Kubala et al., 2008). Our data show that pCPA i.p. did not change locomotor activity. It is possible that an acute administration of pCPA at lower doses does not affect this parameter.

According to Koolhaas et al. (2013) and Takahashi & Miczek (2014), to establish an animal aggressive behavior model, species specific qualitative and quantitative parameters of aggression must be considered. Therefore, when establishing the behaviors to evaluate, we took into account parameters that reconciled both characteristics. We measured aggressive behavior as a quantitative parameter and aggressive latency as a qualitative one (Miczek et al., 2013). Stunder et al. (2015) show decreased aggressive latency, as a parameter of desadaptative aggression, and nonaggressive behaviors in mice treated with pCPA. Our data show that the administration of pCPA increased aggressive behavior and decreased aggressive latency. These findings are consistent with others, in which central 5-HT depletion caused aggression increased (Valzelli et al., 1981; Vergner et al., 1986). This might indicate that our model, with pCPA, was effective to induce aggression, both in a qualitative and quantitative sense. Furthermore, we did not find changes in nonsocial activity and social activity after pCPA administration. 5-HT low-levels might affect social interactions, e.g. in studies where social isolation is used to produce aggressive behavior models (Goodell et al., 2017) or in maternal aggression models (Toth et al., 2012). Thus, this result could be explained by our model and experimental design.

Serotonergic innervation originating in the raphe nuclei towards the different brain structures has one of its main synaptic center in the OB (Locki 1985; Steinfeld et al., 2015). Our results show that after pCPA administration, the concentration of 5-HT in the OB was significantly decreased pointing to higher aggressive behavior. In this way, our model provides evidence to the serotonergic deficiency hypothesis and aggression (Kravitz & Huber, 2003; Miczek et al., 2004; Mongillo et al., 2014; Takahashi et al., 2011; Niederkofler et al., 2016). In addition, 5-HT modification has been associated with changes in its major metabolite, 5-HIAA (Stenfors & Ross, 2004). In humans (Sharma et al., 2021; Stanley et al., 2000) and monkeys (Zajicek et al., 2000), higher aggressiveness and low cerebrospinal 5-HIAA levels were associated. Furthermore, mutual decreased 5-HT and 5-HIAA were observed in models of pCPA aggression (Keleta et al., 2007; Kubala et al., 2008). However, the decrease in 5-HT concentration, following local or systemic administration of substances that affect its release from nerve terminals, does not always affect 5-HIAA concentration in the same way (Auerbach et al., 1989; Kalén at al., 1988). In our model, pCPA administration did not cause significant modifications in the concentration of 5-HIAA. It is possible that systemic depletion did not affect the metabolite as well as 5-HT, because others (Dringenberg et al., 1995; Matte & Tornow, 1978) administered higher doses or performed a chronic treatment (Keleta et al., 2007; Kubala et al., 2008). We also showed that serotonergic metabolism (5-HIAA/5-HT) decreased in treated animals, which is in accordance with previous reported data (Hritcu et al., 2007; Koe & Weissman, 1966). These findings suggest that pCPA may cause alterations in the OB serotonergic innervation, enhancing aggression in our model.

Since pCPA animals exhibited increased aggression and 5-HT decreased, we hypothesized that these differences are accompanied by differences in a key serotonergic gene, TPH2. TPH2 mRNA expression is frequently found in raphe complex neurons in rodents (Malek et al., 2005; Patel et al., 2004; Pelosi et al., 2015;

Walther et al., 2003) and a very low expression has been found in other rats' brain areas, (Patel et al., 2004). Interestingly, we found that pCPA animals showed increased TPH2 mRNA expression in the OB. Therefore, our results provide evidence for TPH2 mRNA expression presence in the OB. It has been also found that after postnatal programming with pCPA, TPH2 mRNA expression decreases in raphe nuclei (Trujillo et al., 2021). In this way, despite that our results are opposite they could be indicating a compensatory mechanism in a brain area receiving 5-HT depleted innervation.

In addition, pCPA decreases the protein expression of GABAA α1 receptors (Wang et al., 2020) and GABAAα1 is expressed in OB (Panzanelli et al., 2005). Interestingly, our results showed a higher expression of GABAAα1 subunit after pCPA administration. These results may indicate increased levels of GABAAα1 subunit mRNA in OB, due to its high synthesis demand caused by the serotonergic decrease as a consequence of pCPA administration.

Conclusion

In this work, we studied aggressive behavior in a rat male model. We concluded that a single and acute pCPA administration produces an increase in aggressive behavior, without affecting locomotion, nonsocial and social activity. We study the OB, a highly innervated serotonergic and GABAergic structure. The pCPA single and acute administration affects the serotonergic activity in OB. We verify TPH2 mRNA expression presence in OB. Moreover, increased GABAAα1 subunit expression mRNA in the OB, may suggest a high synthesis demand, as an alterat result in the OB serotonergic function. Thus, our data provide evidence for the serotonergic deficiency hypothesis of aggression and show OB as a relevant structure to understand neurobiological complexity in aggressive behavior.

Compliance with Ethical Statement

All procedures were made following the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health and the EU (Eighth Edition, 2011) and approved by the Comité Institucional para el Cuidado y Uso de Animales de Laboratorio de la Universidad Nacional de Cuyo (CICUAL UNCuyo) (Aval 82/2016), Mendoza, Argentina.

Limitations

Other brain areas relate to aggressive behavior are not included in the present study.

Data Accessibility Statement

The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are included in the paper.

References

Auerbach, S. B., Minzenberg, M. J., & Wilkinson, L. O. (1989). Extracellular serotonin and 5-hydroxyindoleacetic acid in hypothalamus of the unanesthetized rat measured by in vivo dialysis coupled to high-performance liquid chromatography with electrochemical detection: dialysate serotonin reflects neuronal release. *Brain research*,*499*(2), 281–290.*https://doi.org/10.1016/0006-8993(89)90776-2*Bester-Meredith, J. K., Burns, J. N., Dang, M. N., Garcia, A. M., Mammarella, G. E., Rowe, M. E., & Spatacean, C. F. (2022). Blocking olfactory input alters aggression in male and female California mice (Peromyscus californicus). *Aggressive behavior*, *48*(3), 290–297.*https://doi.org/10.1002/ab.22004*Berger M., Gray, J. A., & Roth, B. L. (2009). The expanded biology of serotonin. Annual review of medicine, 60, 355–366.*https://doi.org/10.1146/annurev.med.60.042307.110802*Bosman, L. W., Rosahl, T. W., & Brussaard, A. B. (2002). Neonatal development of the rat visual cortex: synaptic function of GABAA receptor alpha subunits. The Journal of Physiology, 545(1), 169–181.*https://doi.org/10.1113/jphysiol.2002.026534*Buhot, M. C., Martin, S., & Segu, L. (2000). Role of serotonin in memory impairment. Annals of medicine, 32(3), 210–221.*https://doi.org/10.3109/07853890008998828*Casas, S., García, S., Cabrera, R., Nanfaro, F., Escudero, C., & Yunes, R. (2011). Progesterone prevents depression-like behavior in a model of Parkinson's disease induced by 6- hydroxydopamine in male rats. Pharmacology, biochemistry, and behavior, 99(4),

614–618.*https://doi.org/10.1016/j.pbb.2011.06.012*Cervantes, M. C., & Delville, Y. (2009). Serotonin 5-HT1A and 5-HT3 receptors in an impulsive-aggressive phenotype. *Behavioral neuroscience*, *123*(3), 589–598.*https://doi.org/10.1037/a0015333*Chi, J. D., Odontiadis, J., & Franklin, M. (1999). Simultaneous determination of catecholamines in rat brain tissue by high-performance liquid chromatography. Journal of chromatography. B, Biomedical sciences and applications, 731(2), 361–367.*https://doi.org/10.1016/s0378-4347(99)00255-8*Corthell, J. T., Stathopoulos, A. M., Watson, C. C., Bertram, R., & Trombley, P. Q. (2013). Olfactory bulb monoamine concentrations vary with time of day. Neuroscience, 247, 234–241.*https://doi.org/10.1016/j.neuroscience.2013.05.040*Dringenberg, H. C., Hargreaves, E. L., Baker, G. B., Cooley, R. K., & Vanderwolf, C. H. (1995). p-chlorophenylalanine-induced serotonin depletion: reduction in exploratory locomotion but no obvious sensory-motor deficits. *Behavioural brain research*, *68*(2), 229–237.*https://doi.org/10.1016/0166-4328(94)00174-e*Fibiger, H. C., & Campbell, B. A. (1971). The effect of para-chlorophenylalanine on spontaneous locomotor activity in the rat.*Neuropharmacology*, *10*(1), 25–32.*https://doi.org/10.1016/0028-3908(71)90005-0*Fukunaga, I., Berning, M., Kollo, M., Schmaltz, A., & Schaefer, A. T. (2012). Two distinct channels of olfactory bulb output. Neuron, 75(2), 320–329. https://doi.org/10.1016/j.neuron.2012.05.017 Guillot, P. V., & Chapouthier, G. (1996). Olfaction, GABAergic neurotransmission in the olfactory bulb, and intermale aggression in mice: modulation by steroids. *Behavior genetics*, *26*(5), 497–504.*https://doi.org/10.1007/BF02359754*Goodell, D. J., Ahern, M. A., Baynard, J., Wall, V. L., & Bland, S. T. (2017). A novel escapable social interaction test reveals that social behavior and mPFC activation during an escapable social encounter are altered by post-weaning social isolation and are dependent on the aggressiveness of the stimulus rat. *Behavioural brain research*,*317*, 1–15.*https://doi.org/10.1016/j.bbr.2016.09.025*Harvey, J. D., & Heinbockel, T. (2018). Neuromodulation of Synaptic Transmission in the Main Olfactory Bulb. International journal of environmental research and public health, 15(10), 2194. https://doi.org/10.3390/ijerph15102194 Hernández-Vázquez, F., Garduño, J., & Hernández-López, S. (2018). GABAergic modulation of serotonergic neurons in the dorsal raphe nucleus. Reviews in neurosciences, 30(3), 289–303. https://doi.org/10.1515/revneuro-2018-0014 Hole, K., Johnson, G. E., & Berge, O. G. (1977). 5,7-Dihydroxytryptamine lesions of the ascending 5-hydroxytryptamine pathways: habituation, motor activity and agonistic behavior.*Pharmacology, biochemistry, and behavior*, *7*(3), 205–210.*https://doi.org/10.1016/0091-3057(77)90135-6*Huang, Z., Thiebaud, N., & Fadool, D. A. (2017). Differential serotonergic modulation across the main and accessory olfactory bulbs. The Journal of Physiology, 595(11), 3515–3533. https://doi.org/10.1113/JP273945 Hritcu, L., Clicinschi, M., & Nabeshima, T. (2007). Brain serotonin depletion impairs short-term memory, but not long-term memory in rats. Physiology & behavior, 91(5), 652–657. https://doi.org/10.1016/j.physbeh.2007.03.028 Jéquier, E., Lovenberg, W., & Sjoerdsma, A. (1967). Tryptophan hydroxylase inhibition: the mechanism by which p-chlorophenylalanine depletes rat brain serotonin. Molecular Pharmacology, 3(3), 274–278. Kalén, P., Strecker, R. E., Rosengren, E., & Björklund, A. (1988). Endogenous release of neuronal serotonin and 5-hydroxyindoleacetic acid in the caudate-putamen of the rat as revealed by intracerebral dialysis coupled to high-performance liquid chromatography with fluorimetric detection. *Journal of neurochemistry*, *51*(5), 1422–1435. https://doi.org/10.1111/j.1471-4159.1988.tb01107.x Keleta, Y. B., Lumia, A. R., Anderson, G. M., & McGinnis, M. Y. (2007). Behavioral effects of pubertal anabolic androgenic steroid exposure in male rats with low serotonin. *Brain research*, *1132*(1), 129–138. https://doi.org/10.1016/j.brainres.2006.10.097 Khan, I. A., & Thomas, P. (2004). Aroclor 1254 inhibits tryptophan hydroxylase activity in the rat brain. Archives of toxicology, 78(6), 316–320. https://doi.org/10.1007/s00204-003-0540-1 Koe, B.K. and Weissman, A. (1966) p-Chlorophenylalanine: a specific depletion of brain serotonin. J. Pharmacol. Exp. Ther. 154: 499–516. Kohlert, J. G., Mangan, B. P., Kodra, C., Drako, L., Long, E., & Simpson, H. (2012). Decreased aggressive and locomotor behaviors in Betta splendens after exposure to fluoxetine. Psychological Reports, 110(1), 51–62. https://doi.org/10.2466/02.13.PR0.110.1.51-62 Koolhaas, J. M., Coppens, C. M., de Boer, S. F., Buwalda, B., Meerlo, P., & Timmermans, P. J. (2013). The resident-intruder paradigm: a standardized test for aggression, violence, and social stress. Journal of visualized experiments: JoVE, (77), e4367. https://doi.org/10.3791/4367 Kravitz, E. A., & Huber, R. (2003). Aggression in invertebrates. Current opinion in neurobiology, 13(6), 736–743. https://doi.org/10.1016/j.conb.2003.10.003 Kubala, K. H., McGinnis, M. Y., Anderson, G. M., & Lumia, A. R. (2008). The effects of an anabo-

lic androgenic steroid and low serotonin on social and non-social behaviors in male rats. *Brain research,1232*, 21–29.*https://doi.org/10.1016/j.brainres.2008.07.065*Lesch, K. P., & Waider, J. (2012). Serotonin in the modulation of neural plasticity and networks: implications for neurodevelopmental disorders. Neuron, 76(1), 175–191. https://doi.org/10.1016/j.neuron.2012.09.013 Linster, C., & Fontanini, A. (2014). Functional neuromodulation of chemosensation invertebrates. Current opinion in neurobiology, 29, 82–87. https://doi.org/10.1016/j.conb.2014.05.010 Lucki I. (1998). The spectrum of behaviors influenced by serotonin.*Biological psychiatry*, *44*(3), 151–162.*https://doi.org/10.1016/s0006-3223(98)00139-5*Malek, Z. S., Dardente, H., Pevet, P., & Raison, S. (2005). Tissue-specific expression of tryptophan hydroxylase mRNAs in the rat midbrain: anatomical evidence and daily profiles. The European journal of neuroscience, 22(4), 895–901. https://doi.org/10.1111/j.1460-9568.2005.04264.x Matte, A. C., & Tornow, H. (1978). Parachlorophenylalanine produces dissociated effects on aggression "emotionality" and motor activity.*Neuropharmacology*, *17*(8), 555–558. https://doi.org/10.1016/0028-3908(78)90147-8 McLean, J. H., & Shipley, M. T. (1987). Serotonergic afferents to the rat olfactory bulb: I. Origins and laminar specificity of serotonergic inputs in the adult rat. The Journal of neuroscience: the official journal of the Society for Neuroscience, 7(10), 3016–3028. https://doi.org/10.1523/JNEUROSCI.07-10-03016.1987 Miczek, K. A., de Boer, S. F., & Haller, J. (2013). Excessive aggression as a model of violence: a critical evaluation of current preclinical methods. Psychopharmacology, 226(3), 445–458. https:// doi.org/10.1007/s00213-013-3008-x Miczek, K. A., Fish, E. W., De Bold, J. F., & De Almeida, R. M. (2002). Social and neural determinants of aggressive behavior: pharmacotherapeutic targets at serotonin, dopamine, and gamma-aminobutyric acid systems. Psychopharmacology, 163(3-4), 434–458. https://doi.org/10.1007/s00213-002-1139-6 Miguez, J., Martin, F., & Aldegunde, M. (1991). Differential effects of pinealectomy on amygdala and hippocampus serotonin metabolism. Journal of pineal research, 10(2), 100–103. https:// doi.org/10.1111/j.1600-079x.1991.tb00017.x Mittal, R., Debs, L. H., Patel, A. P., Nguyen, D., Patel, K., O'Connor, G., Grati, M., Mittal, J., Yan, D., Eshraghi, A. A., Deo, S. K., Daunert, S., & Liu, X. Z. (2017). Neurotransmitters: The Critical Modulators Regulating Gut-Brain Axis. Journal of cellular physiology, 232(9), 2359–2372. https://doi.org/10.1002/jcp.25518 Moeller, F. G., Dougherty, D. M., Swann, A. C., Collins, D., Davis, C. M., & Cherek, D. R. (1996). Tryptophan depletion and aggressive responding in healthy males. *Psychopharmacology*, *126*(2), 97–103.*https://doi.org/10.1007/BF02246343*Mongillo, D. L., Kosyachkova, E. A., Nguyen, T. M., & Holmes, M. M. (2014). Differential effects of chronic fluoxetine on the behavior of dominant and subordinate naked mole-rats. Behavioral brain research, 258, 119–126. https://doi.org/10.1016/j.bbr.2013.10.023 Muroy, S. E., Long, K. L., Kaufer, D., & Kirby, E. D. (2016). Moderate Stress-Induced Social Bonding and Oxytocin Signaling are Disrupted by Predator Odor in Male Rats. Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology, 41(8), 2160–2170. https://doi.org/10.1038/npp.2016.16 Muzerelle, A., Scotto-Lomassese, S., Bernard, J. F., Soiza-Reilly, M., & Gaspar, P. (2016). Conditional anterograde tracing reveals distinct targeting of individual serotonin cell groups (B5-B9) to the forebrain and brainstem. Brain structure & function, 221(1), 535–561. https://doi.org/10.1007/s00429-014-0924-4 Näslund, J., Studer, E., Pettersson, R., Hagsäter, M., Nilsson, S., Nissbrandt, H., & Eriksson, E. (2015). Differences in Anxiety-Like Behavior within a Batch of Wistar Rats Are Associated with Differences in Serotonergic Transmission, Enhanced by Acute SRI Administration, and Abolished By Serotonin Depletion. *The international journal of neuropsychopharmacology*, *18*(8), pyv018.*https://doi.org/10.1093/ijnp/pyv018*Niederkofler, V., Asher, T. E., Okaty, B. W., Rood, B. D., Narayan, A., Hwa, L. S., Beck, S. G., Miczek, K. A., & Dymecki, S. M. (2016). Identification of Serotonergic Neuronal Modules that Affect Aggressive Behavior. *Cell reports*, *17*(8), 1934–1949.*https://doi.org/10.1016/j.celrep.2016.10.063*Panzanelli, P., Perazzini, A.-Z., Fritschy, J.-M., & Sassoè-Pognetto, M. (2005). Heterogeneity of γ-aminobutyric acid types A receptors in mitral and tufted cells of the rat main olfactory bulb. Journal of Comparative Neurology, 484(1), 121–131. doi:10.1002/cne.20440 Pfaffl M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic acids research, 29(9), e45. https://doi.org/10.1093/nar/29.9.e45 Pelosi, B., Pratelli, M., Migliarini, S., Pacini, G., & Pasqualetti, M. (2015). Generation of a Tph2 Conditional Knockout Mouse Line for Time- and Tissue-Specific Depletion of Brain Serotonin. PloS one, 10(8), e0136422. https://doi.org/10.1371/journal.pone.0136422 Popova, N. K., Gilinsky, M. A., Amstislavski, T. G., Morosova, E. A., Seif, I., & De Maeyer, E. (2001). Regional serotonin metabolism in

the brain of transgenic mice lacking monoamine oxidase A. Journal of neuroscience research, 66(3), 423–427. https://doi.org/10.1002/jnr.1234 Raghuveer, K., Sudhakumari, C. C., Senthilkumaran, B., Kagawa, H., Dutta-Gupta, A., & Nagahama, Y. (2011). Gender differences in tryptophan hydroxylase-2 mRNA, serotonin, and 5-hydroxytryptophan levels in the brain of catfish, Clarias gariepinus, during sex differentiation. General and comparative endocrinology, 171(1), 94–104. https://doi.org/10.1016/j.ygcen.2010.12.003 Scott, J. W., Wellis, D. P., Riggott, M. J., & Buonviso, N. (1993). Functional organization of the main olfactory bulb. Microscopy research and technique, 24(2), 142–156. https://doi.org/10.1002/jemt.1070240206 Ogawa, S., Tsuchimine, S., & Kunugi, H. (2018). Cerebrospinal fluid monoamine metabolite concentrations in depressive disorder: A meta-analysis of historic evidence. *Journal of psychiatric research*, *105*, 137–146.*https://doi.org/10.1016/j.jpsychires.2018.08.028*Stanley, B., Molcho, A., Stanley, M., Winchel, R., Gameroff, M. J., Parsons, B., & Mann, J. J. (2000). Association of aggressive behavior with altered serotonergic function in patients who are not suicidal.*The American journal of psychiatry*, *157*(4), 609–614.*https://doi.org/10.1176/appi.ajp.157.4.609*Si, Y., Wei, W., Chen, X., Xie, X., Guo, T., Sasaki, Y., Zhang, Y., Wang, L., Zhang, F., & Feng, S. (2022). A comprehensive study on the relieving effect of *Lilium brownii* on the intestinal flora and metabolic disorder in *p*-chlorphenylalanine induced insomnia rats.*Pharmaceutical biology*, *60*(1), 131–143.*https://doi.org/10.1080/13880209.2021.2019283*Steinfeld, R., Herb, J. T., Sprengel, R., Schaefer, A. T., & Fukunaga, I. (2015). Divergent innervation of the olfactory bulb by distinct raphe nuclei. The Journal of comparative neurology, 523(5), 805–813. https://doi.org/10.1002/cne.23713 Stenfors, C., & Ross, S. B. (2004). Changes in extracellular 5-HIAA concentrations as measured by in vivo microdialysis technique in relation to changes in 5-HT release. *Psychopharmacology,172*(2), 119–128. https://doi.org/10.1007/s00213-003-1736-z Strüder, H. K., & Weicker, H. (2001). Physiology and pathophysiology of the serotonergic system and its implications on mental and physical performance. Part II. International journal of sports medicine, 22(7), 482–497.https://doi.org/10.1055/s-2001-17606 Takahashi, A., Quadros, I. M., de Almeida, R. M., & Miczek, K. A. (2012). Behavioral and pharmacogenetics of aggressive behavior. Current topics in behavioral neurosciences, 12, 73–138. https://doi.org/10.1007/7854_2011_191 Takahashi, A., Quadros, I. M., de Almeida, R. M., & Miczek, K. A. (2011). Brain serotonin receptors and transporters: initiation vs. termination of escalated aggression. Psychopharmacology, 213(2-3), 183–212. https://doi.org/10.1007/s00213-010-2000-y Takahashi A and Miczek KA. (2014) Neurogenetics of Aggressive Behavior – Studies in Rodents. Curr Top Behav Neurosci. 2014; 17: 3–44.https://doi.org/10.1007/7854_2013_263 Toth, M., Tulogdi, A., Biro, L., Soros, P., Mikics, E., & Haller, J. (2012). The neural background of hyper-emotional aggression induced by post-weaning social isolation. Behavioral brain research, 233(1), 120–129. https://doi.org/10.1016/j.bbr.2012.04.025 Trujillo, V., Valentim-Lima, E., Mencalha, R., Carbalan, Q., Dos-Santos, R. C., Felintro, V., Girardi, C., Rorato, R., Lustrino, D., Reis, L. C., & Mecawi, A. S. (2021). Neonatal Serotonin Depletion Induces Hyperactivity and Anxiolytic-like Sex-Dependent Effects in Adult Rats.*Molecular neurobiology*, *58*(3), 1036–1051.*https://doi.org/10.1007/s12035-020-02181-0*Urban, N. N., & Arevian, A. C. (2009). Computing with dendrodendritic synapses in the olfactory bulb. Annals of the New York Academy of Sciences, 1170, 264–269. https://doi.org/10.1111/j.1749-6632.2009.03899.x van Erp, A. M., & Miczek, K. A. (2000). Aggressive behavior, increased accumbal dopamine, and decreased cortical serotonin in rats. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *20*(24), 9320–9325.*https://doi.org/10.1523/JNEUROSCI.20-24-09320.2000*Vergnes, M., Depaulis, A., Boehrer, A., & Kempf, E. (1988). Selective increase of offensive behavior in the rat following intrahypothalamic 5,7-DHT-induced serotonin depletion. *Behavioural brain research,29*(1-2), 85–91.*https://doi.org/10.1016/0166-4328(88)90055-1*Vergnes, M., & Kempf, E. (1982). Effect of hypothalamic injections of 5,7-dihydroxytryptamine on elicitation of mouse-killing in rats.*Behavioral brain research*, *5*(4), 387–397. https://doi.org/10.1016/0166-4328(82)90042-0 Wang, M., Li, N., Jing, S., Wang, C., Sun, J., Li, H., Liu, J., & Chen, J. (2020). Schisandrin B exerts hypnotic effects in PCPA-treated rats by increasing hypothalamic 5-HT and γ-aminobutyric acid levels.*Experimental and therapeutic medicine*, *20*(6), 142.*https://doi.org/10.3892/etm.2020.9271*Walther, D. J., & Bader, M. (2003). A unique central tryptophan hydroxylase isoform. Biochemical pharmacology, 66(9), 1673–1680. https://doi.org/10.1016/s0006-2952(03)00556-2 Yu, H. L., Chen, Z. J., Zhao, J. W., Duan, S. R., & Zhao, J. K. (2019). Olfactory Impairment and Hippocampal Volume in a Chinese MCI Clinical Sample. Alzheimer's disease and associated disorders,

33(2), 124–128. https://doi.org/10.1097/WAD.0000000000000305 Zajicek, K. B., Price, C. S., Shoaf, S. E., Mehlman, P. T., Suomi, S. J., Linnoila, M., & Higley, J. D. (2000). Seasonal variation in CSF 5-HIAA concentrations in male rhesus macaques.*Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *22*(3), 240–250.*https://doi.org/10.1016/S0893-133X(99)00097-4*Zepf, F. D., Stadler, C., Demisch, L., Schmitt, M., Landgraf, M., & Poustka, F. (2008). Serotonergic functioning and trait-impulsivity in attention-deficit/hyperactivity-disordered boys (ADHD): influence of rapid tryptophan depletion. *Human psychopharmacology*,*23*(1), 43–51.*https://doi.org/10.1002/hup.896*Zill, P., Büttner, A., Eisenmenger, W., Möller, H. J., Ackenheil, M., & Bondy, B. (2007). Analysis of tryptophan hydroxylase I and II mRNA expression in the human brain: a post-mortem study. Journal of psychiatric research, 41(1-2), 168–173. https://doi.org/10.1016/j.jpsychires.2005.05.004 Zhang, J. H., Araki, T., Sato, M., & Tohyama, M. (1991). Distribution of GABAA-receptor alpha 1 subunit gene expression in the rat forebrain.*Brain research. Molecular brain research*, *11*(3-4), 239–247.*https://doi.org/10.1016/0169-328x(91)90032-s*

Figures and Tables Legends

Table 1: Real Time PCR Primer Design.

Specific primers designed for rat gene sequence amplification were selected for real-time PCR assay.

Table 2: pCPA effects in locomotor activity, Nonsocial activity and Social activity.

Locomotor activity results are expressed as mean ± SEM of distanced travel in centimeters. Nonsocial activity and social activity results are expressed in mean ± SEM of time in seconds. Control (N=10) and pCPA (N=10).

Table 3: pCPA effects in 5-HT, 5-HIAA and turnover %5-HIAA/5-HT.

Content determination by HPLC in the OB 5-HT and 5-HIAA results are expressed in ± SEM of pg/mg protein. Turnover 5-HIAA/5-HT results are expressed in mean ± SEM of the 5-HIAA/5-HT pg/mg protein percentage ratio. Control (N=10) and pCPA (N=10).

Figure 1: A- pCPA effects in Aggressive Behavior. Results are expressed in the mean ± SEM of several events. B- pCPA effects in Aggressive Latency. Results are expressed in mean ± SEM of latency in time of the first aggressive behavior. Control (N=10) and pCPA (N=10). ***p <0.001; ****p <0.0001 for "t" Test.

Figure 2: A- pCPA effects in TPH2 gene expression in the OB. Results are expressed in mean ± SEM of TPH2 relative expression units. B- pCPA effects GABAAα1 mRNA expression in the OB. Results are expressed in mean ± SEM of GABAAα1 relative expression units. Control (N=10) and pCPA (N=10). *p <0.05 for t-Test.

mRNAs		Primer Sequence	Gene Bank accession No	Amplicon size	Annealing temperature	Anneling exon
TPH2	Sense	CGGCGAAGAAGTTCTGAAGT	NM_173839.2	164	60.6	Exon 2
	Antisense	AACCACGGCACATCCTCTA				Exon 3
$GABA_{An1}$	Sense	CGGCTGAACAACCTGATGG	NM_183326.2	163	60.6	Exon 4
	Antisense	ATTCGGCTCTCACAGTCAAC				Exon 5
S16	Sense	TCCAAGGGTCCGCTGCAGTC	NM_001169146.1	100	60	Exon 1
	Antisense	CATTCACCTTGATGAGCCCATT				Exon 2

	Control mean ± SEM	pCPA mean ± SEM	Statistics
Locomotor activity	1338 ± 113.8	1452 ± 113.4	p=0.3296; t=1.004; df=18
Nonsocial activity	382.8 ± 67.99	324.9 ± 47.99	p=0.1746; t=1.417; df=18
Social activity	160.6 ± 18.48	179.1 ± 21.49	p= 0.1957; t=1.347; df=18

	Control mean ± SEM	pCPA mean ± SEM	Statistics
5-HT	85.51± 9.129	44.38 ± 10.17	p=0.0078 t= 2.954; df=18
5-HIIA	0.834 ± 0.041	0.86 ± 0.084	p= 0.7982; t= 0.2591, df= 18
Turnover % 5-HIAA/5HT	1.035 ± 0.138	2.577 ± 0.447	p=0.0232 ; t= 2.634; df=18