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Research Article

Para-Chlorophenyl Alanine Induces Aggressive Behavior by Serotonin Depletion in Male Rats and Increases Tryptophan Hydroxylase two 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 the 5-HT synthesis, and para- chlorophenyl alanine (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 the olfactory bulb, considered a relevant structure in rodents for establishing social interactions, including aggressive behavior. However, the relationship between the olfactory bulb and aggression in a pCPA 5-HT depletion model has not been studied.

Moreover, receptor subunit GABAα1 has been found in the olfactory bulb, and 5-HT depletion could affect GABAA receptor expression in different brain areas. Thus, we aimed to evaluate aggressive behavior, serotonergic activity, and the TPH2 and GABAAα1 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. The olfactory bulb was obtained, and neurochemical and molecular techniques were used to measure 5-HT, 5-HIAA, TPH2, and GABAAα1 mRNA expression, respectively. pCPA administration increased aggressive behavior parameters without affecting locomotion, non-social or social interaction. 5-HT levels were decreased after pCPA administration and its turnover rate, although there were no significant changes in 5-HIAA. TPH2 mRNA expression was increased. GABAAα1 mRNA expression was increased in the olfactory bulb. Our results apport evidence to the serotonergic deficiency hypothesis of aggression and highlight the olfactory bulb's Role as an essential structure for understanding aggressive behavior neurobiological complexity.

Keywords: Rat, Raphe nuclei, Olfactory bulb, Social interaction, Resident intruder test

Introduction

Aggressiveness is considered a social behavior since it requires at least two subjects and can be either defensive or offensive. There are similarities in aggressive neurobiology among primates, humans, and rodents [1,2]. The aggressive spectrum displayed depends on the specie, the sex, the stimuli, and the contextual contingency [1-3]. According to the qualitative and quantitative features,

aggressiveness in rodents could be considered adaptive or maladaptive [4]. In their natural environment, rodents are powerfully aggressive in defending their food resources, reproductive possibilities, and territory [5]. The Resident Vs. Intruder Test (RVI) is the most validated paradigm to measure this interaction [3].

Serotonin (5-HT) plays an essential role in social behavior. Among others, modulates several behaviors, such as copula, postnatal care, adolescent social playing, and maternal and territorial aggressiveness [5-9]. Many systems are involved in aggressive behavior *Chamero, et al.,* (2011), *Carrillo, et al.,* (2009), *Duke, et al.,* (2013) Although, serotonergic deficiency hypothesis establishes an inverse relationship between the serotonergic system activity and aggressive behavior [1,2,10-13] *Miczek, et al.,* (2004). In rodent models, there are several ways to generate aggressiveness by decreasing 5-HT: neurotoxicity, olfactory bulbectomy, social isolation, and pharmacological depletion [14-17]. Para-chlorophenylalanine (pCPA) is an irreversible TPH enzyme inhibitor. An intraperitoneal pCPA administration causes an acute decrease within the central serotonergic system [14] *Jáquier, et al.,* (1967). It has been used to generate aggression models [18,19] *Jáquier et al.,* (1967).

Serotonin-producing neurons in the raphe complex project their axons to the cortex, amygdala, hippocampus, basal ganglia, thalamus, hypothalamus, and Olfactory Bulb (OB) [20] *Mazerolle, et al.,* (2016). OB is an important serotonergic innervated structure [21-23], and the primary sensory information used by rodents to start social interactions, such as aggressive behavior, is olfaction [24]. Moreover, [25] showed that 5-HT synthesis inhibition in OB rats caused increased aggressiveness, whereas enhancement transmission suppressed aggressive behavior. The first sensory center that processes odor information is the OB, a structure considered when studying territorial aggression [26]. 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 [27]. TPH gene has two isoforms [28] while the TPH1 isoform is mainly expressed outside the blood-brain barrier, the TPH2 isoform is expressed in the cerebral tissue, and its mRNA expression has so far been primarily described in raphe complex neurons [28-30] *Patel, et al.,* (2004). A human brain post-mortem study showed TPH2 expression in the cortex, thalamus, hypothalamus, hippocampus, amygdala, cerebellum, and raphe nuclei [31]. A study using catfishes [32] reported TPH2 mRNA expression in OB. Furthermore, *Patel, et al.,* (2004) showed that TPH2 mRNA expression in rat brains revealed a weak signal in the OB.

In OB, principal neurons and local interneurons have GABAA receptors with different subunit components [33]. Remarkably, the presence of GABAA receptor subunit α1mRNA in the OB has been described [34] and is the most common GABAAα subunit in adult rodent neurons [35]. In addition, it has been reported that 5-HT depletion could affect GABAA receptor expression in different brain areas [36,37]. We aimed to evaluate aggressive behavior and 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 (60-70lumens). Food and water were available ad libitum.

RVI test requires a resident subject and an intruder. Residents were Sprague-Dawley male rats of 60days and 350g (average weight). Intruders were Sprague-Dawley male rats of 50days and 280g (average weight). Resident subjects were divided into two groups (n=10). Experimental subjects were injected with pCPA and returned to their home cage for six days; control subjects were injected with a vehicle and returned to their home cage simultaneously. After the RVI test, residents were euthanized, and OB was removed for posterior analysis [38]. Intruder subjects remained housed in groups of 3 until the test and were euthanized.

Experimental Design

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

Behavioral Testing

To assess aggressive behaviors, RVI was used. RVI test was applied according to [38] 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 six days after pCPA administration, between 15:00h and 18:00h [39]. To verify that residents were heavier than intruders, the animal's weights before the test. The test was carried out in a wooden box with wood chips from the resident's home cage. The box was cleaned after each test with ethanol (10%) [40]. 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. Six hundred 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 behav-

ior, as an event of the chase, moving towards, upright posture and false mount, bite, clinch, clinch attack, lateral threat and kept 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). 10μg of total RNA was reverse transcribed at 37°C using random hexamer primers and Moloney murine leukemia virus retrotranscriptase (Invitrogen-Life Technologies, Buenos Aires, Argentina) in a 20μL reaction mixture. The RNA was first denatured at 70°C for 5min in the presence of 2.5μg of random hexamer primers (Invitrogen). For the subsequent RT reaction, the following mixture was added: RT buffer [50mM Tris-HCl (pH 8.4), 75 mM KCl, 3mM MgCl2], 0.5mM dNTPs, 5mM DTT, 200 units M-MLV Reverse Transcriptase (Invitrogen). The reaction was incubated at 37°C for 50 min.; next, the reaction was inactivated by heating at 70°C for 15min. The cDNA was stored at-20°C. The mRNA levels of TPH2 and GABAAα1 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).

Table 1: Real-Time PCR Primer Design. Specific primers for rat gene sequence amplification were selected for real-time PCR assay.

mRNAs		Primer Sequence	Gene Bank accession No	Amplicon size	Annealing temperature	Anneling exon
TPH2	Sense	CGGC-GAAGAAGTTCT-GAAGT	NM_173839.2	164	60.6	Exon 2
	Antisense	AACCACGGCA-CATCCTCTA				Exon 3
GABAAα1	Sense	CGGCTGAA-CAACCTGATGG	NM_183326.2	163	60.6	Exon 4
	Antisense	ATTCGGCTCTCA-CAGTCAAC				Exon 5
S16	Sense	TCCAAGGGTC-CGCTGCAGTC	NM_001169146.1	100	60	Exon 1
	Antisense	CATTCACCTTGAT-GAGCCCATT				Exon 2

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 20μL. The reaction mixture consisted of 2μL of 10×PCR Buffer, 1μL of 50mM MgCl2, 0.4μL of 10 mM dNTP Mix (Invitrogen), 1μL of 20× Eva Green, 0.25μL of 5U/μL Taq DNA Polymerase (Invitrogen) 0.1μL of each 2.5mM primer (forward and reverse. primers) and 10μL of diluted cDNA. The PCR reactions were performed under the conditions described in Table 1. Melt curve analysis was used to determine whether a 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 [41]. 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. 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 5min at 95ºC followed by a three-step scheme: 30 s at 95ºC, 30 s at the annealing temperature shown above for each primer pair, and a final step at 72ºC 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 [42]. Briefly, the tissue was collected in 400μl of 0.2N 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. 5-HT and 5-HIAA were expressed as picograms per milligram of to-

tal protein. 10µl 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 650mV, 0.5nA; model LC-4C, BAS, West Lafayette, IN, USA). The mobile phase, containing 0.05 M NaH2PO4, 1.0mM 1-octane sulfonic acid, 0.27mM 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 sample's respective peak area and elution time with a reference standard. The quantification was performed using a calibration curve for each neurotransmitter (Program Chrom Pass, 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. The 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±SEM of 10 rats per experimental group. All data were analyzed using Statistic's software application (Stat Soft, 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 compared to the control group. Moreover, pCPA did not affect non-social activity (p= 0.1746; t= 1.417; df= 18) and social activity (p= 0.1957; t= 1.347; df= 18) (Table 2).

Table 2: pCPA effects on locomotor, Non-social activity, and social activity. Locomotor activity results are expressed as mean ± SEM of distanced travel in centimeters. Non-social and social activity results are expressed in mean ± SEM of time in seconds: control (N=10) and pCPA (N=10).

	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

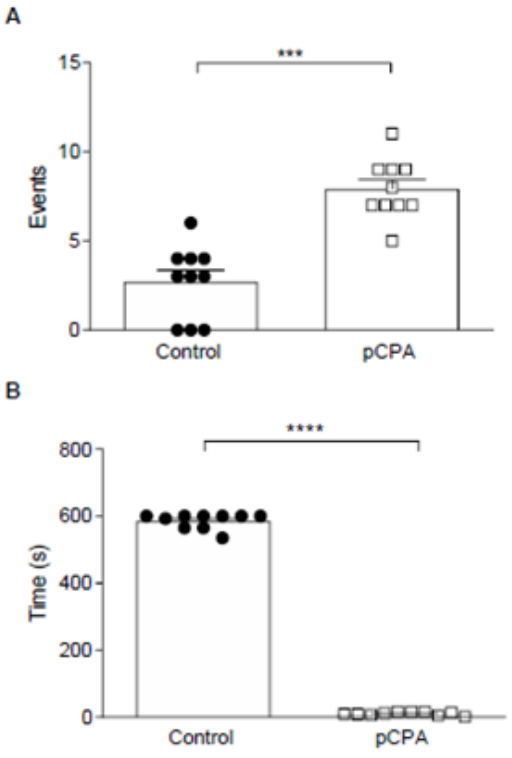

Figure 1: A- pCPA effects in Aggressive Behavior. Results are expressed in the mean ± SEM of several events. B- pCPA effects in Aggressive Latency. Results are expressed in mean ± SEM of latency during the first aggressive behavior.
Note*: Control (N=10) and pCPA (N=10). ***p <0.001; ****p <0.0001 for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

However, pCPA induced a significant increase in aggressive behavior concerning 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).

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).

	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

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).

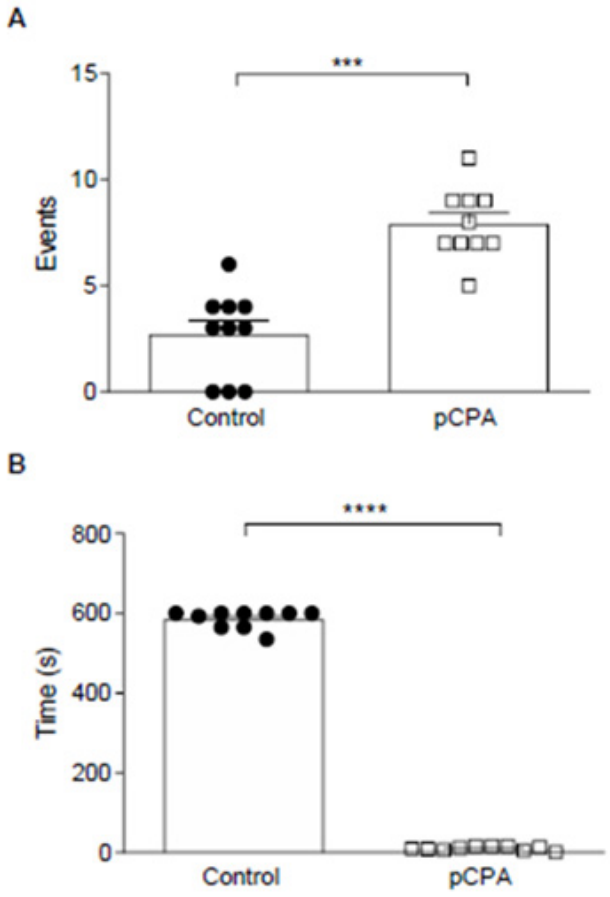

Figure 2: A- pCPA effects in TPH2 gene expression in the OB. Results are expressed in mean ± SEM of TPH2 relative expression units. B- pCPA affects GABAAα1 mRNA expression in the OB.
Note*: Results are expressed in mean ± SEM of GABAAα1 relative expression units. Control (N=10) and pCPA (N=10). *p <0.05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

Discussion

The initiation, maintenance, and termination of aggressive behavior activate complex neurobiological circuits, among which the serotonergic system is intensely involved [2,3]. In the current study, using an 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 discarding locomotor activity alterations is necessary when evaluating social interaction. [3,43]. Alocomotor activity alteration in the resident could prevent the correct measurement of aggressive behavior, particularly the aggression latency parameter. Interestingly, 5,7- dihydroxytryptamine lesions, which deplete central 5-HT, did not affect locomotor activity [44] *Vergner, et al.,* (1988). Nonetheless, previous works utilizing pCPA 1000 mg/kg doses found a substantial decrease in locomotor activity [45,46]. Significantly reduced locomotor activity was also seen following chronic treatment with a lower dosage of pCPA (100 mg/kg) [18,19]. Our data show that pCPA i.p. did not change locomotor activity. An acute administration of pCPA at lower doses does not affect this parameter.

According to [3,38,] species-specific qualitative and quantitative aggression parameters must be considered to establish an animal aggressive behavior model. Therefore, we considered parameters that reconciled both characteristics when evaluating behaviors. We measured aggressive behavior as a quantitative parameter and aggressive latency as a qualitative one [43,47]. Show decreased aggressive latency as a parameter of des adaptative aggression and nonaggressive behaviors in mice treated with pCPA. Our data show that administering pCPA increased aggressive behavior and decreased aggressive latency. These findings are consistent with others in which central 5-HT depletion caused an aggression increase *Valzelli, et al.,* (1981)*, Vergner, et al.,* (1986). indicating that our model, with pCPA, was adequate to induce aggression, both in a qualitative and quantitative sense. Furthermore, we did not find changes in non-social activity and social activity after the pCPA administration. 5-HT low levels might affect social interactions, e.g., in studies where social isolation produces aggressive behavior models [48] or maternal aggression models [49]. 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 centers in the OB [23] *Locki, et al.,* (1985). Our results show that after pCPA administration, the concentration of 5-HT in the OB was significantly decreased, pointing to higher aggressive behavior. Our model provides evidence for the serotonergic deficiency hypothesis and aggression [1,2,11,12] *Miczek, et al.,* (2004). In addition, 5-HT modification has been associated with changes in its major metabolite, 5-HIAA [50]. In humans [51] *Sharma, et al.,* (2021) and monkeys [52], 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 [18,19]. However, the decrease in 5-HT concentration, following local or systemic administration of substances that affect their release from nerve terminals, does not always affect 5-HIAA concentration similarly [53]. In our model, pCPA administration did not cause significant modifications in the concentration of 5-HIAA. Probably, systemic depletion did not affect the metabolite as well as 5-HT because others [45,46] administered higher doses or performed a chronic treatment [18,19]. Under previously reported data, we also showed that serotonergic metabolism (5-HIAA/5-HT) decreased in treated animals [19,24]. 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 differences in a critical serotonergic gene, TPH2 accompany these differences. TPH2 mRNA expression is frequently found in raphe complex neurons in rodents [28-30] *Patel, et al.,* (2004), and a shallow expression has been found in other rats' brain areas, *Patel, et al.,* (2004). Interestingly, pCPA animals showed increased TPH2 mRNA expression in the OB. Therefore, our results provide evidence for the TPH2 mRNA expression presence in the OB. It has also been found that after postnatal programming with pCPA, TPH2 mRNA expression decreases in raphe nuclei [54-72]. In this way, despite our opposite results, they could indicate a compensatory mechanism in a brain area receiving 5-HT depleted innervation.

In addition, pCPA decreases the protein expression of GABAA α1 receptors [37], and GABAAα1 is expressed in OB [33]. Interestingly, our results showed a higher expression of the 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 due to 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, non-social 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 alternate 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 understanding 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

The present study does not include other brain areas related to aggressive behavior.

Data Accessibility Statement

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

Conflict of Interests

The authors declared no potential conflict of interest concerning this article's research, authorship, and publication.

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