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# Intrauterine Exposure to Chronic 22 kHz Sound Affects Inhibitory Avoidance and Serotonergic Parameters in Forebrain Areas of Dams and Rat Offspring

**Patrícia da Silva Oliveira[1], Ariomester Nascimento Daniel[1], Polliana Toledo Nunes[1], Karina Abdo Costa[1], Hani Camille Yehia[1,2], Angela Maria Ribeiro[1,3*]**

[1]Programa de Pós-Graduação em Neurociências, Laboratório de Neurociências Comportamental e Molecular, LaNeC, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil
[2]Departamento de Engenharia Eletrônica, Escola de Engenharia, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil
[3]Departamento de Bioquímica e Imunologia, ICB and Departamento de Psicologia, FaFiCH, Laboratório de Neurociências Comportamental e Molecular, LaNeC, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil
Email: *angelamr@icb.ufmg.br

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## Abstract

**In the present study we evaluated the effects of chronic exposure to sounds at 22 kHz during pregnancy on the central serotonergic and behavioral parameters in Wistar rat dams after the suckling period and on their male rat offspring. In addition, we also assessed the effects of an acute 22 kHz sound, associated with the chronic intrauterine exposure, on the emotional responses of adult offspring. The primary hypothesis was that experiencing 22 kHz stimuli during an early stage of development would interfere with brain serotonergic parameters and, later, with the adult rat's defensive responses. The corollary question was whether a 22 kHz sound exposure would differentially affect inhibitory avoidance and escape responses and central serotonergic parameters. Female rats were divided into four groups: non-pregnant control; non-pregnant chronic exposure; pregnant control; and pregnant chronic exposure. Male offspring were divided into four groups: chronic intrauterine sound exposure; acute sound exposure in adulthood; chronic intrauterine exposure with acute exposure in adulthood; and no exposure. Chronic sound exposure af-**

*Corresponding author.

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**fected inhibitory avoidance and serotonergic parameters in female rats. For offspring, there was an interaction between chronic and acute sound exposure effects on inhibitory avoidance response but not on escape response. There were significant effects of chronic intrauterine exposure on serotonin turnover in the hippocampus and PFC of females. For offspring, the turnover was increased by chronic exposure only in PFC, and in amygdala it was increased by acute exposure. These results illuminate the potential of an early acoustic sound exposure for causing central serotonergic and emotional behavioral changes that can persist into later periods of life.**

## Keywords

**Intrauterine, 22 kHz Sound Exposure, Defensive Responses, Serotonergic Parameters, Adult Offspring**

## 1. Introduction

Rats can vocalize in the sonic and ultrasonic wide range. Their ultrasonic vocalizations (USV) range from 20 to about 100 kHz [1]. They are also able to detect such ultrasonic signals. Within this wide range, the 22 kHz call seems to be related to aversion occurring in response to immediate and potential environmental threats [2]-[4]. These vocalizations are carried out in many situations in which an evident aversive component is present, for example, exposure to a predator [5] or a social fight between males [6]. Some authors [7] [8] demonstrated that 22 kHz USVs induce activation in brain areas related to motivation and emotion (e.g., amygdala). Freezing behavior in response to 22 kHz USV is paralleled by increased neuronal activity in brain areas regulating fear and anxiety, such as the amygdale and periaqueductal gray [4]. Although the terms anxiety and fear are often used interchangeably; there is evidence that they can be differentiated. For instance, there are data that suggest that USV was found useful for differentiating fear and anxiety in rats [9]. Using behavioral and pharmacological approaches the authors showed evidence that rat 22 kHz USV can be considered to be a correlate of anxiety rather than of fear. Accordingly, 22 kHz exposure might be an interesting tool for studying behavioral and neurobiological aspects of emotion, such as the defensive responses related to anxiety and fear.

The primary hypothesis was that experiencing 22 kHz stimuli during an early stage of development would interfere with brain serotonergic parameters and, later, with the adult rat's defensive responses. The corollary question was whether a 22 kHz sound exposure would differentially affect inhibitory avoidance and escape responses and central serotonergic parameters. The rationale of this idea is that a biological phenomenon known as programming can be triggered when an animal, e.g., a human [10] or a rodent [11], is exposed to a stimulus in a critical period of development. This promotes anatomical, physiological, metabolic and neuropsychological adaptive changes that can persist into later periods of life [11] [12].

Certain environmental conditions experienced during an early period of animal development induce programming. Maternal stress [13]-[17] is one such condition. In addition, it is known that exposure to noise during pregnancy influences neurogenesis and thickness in the brain cortex of rat pups [18]. However, to date there is no study on the effects of intrauterine 22 kHz sound exposure on dam and on adult offspring emotional and brain neurochemical aspects, when the individuals are confronted with a situation of threat which might induce defensive behaviors, such as inhibitory avoidance and escape.

Some authors [19] developed an animal model that allowed the same rat to perform an inhibitory avoidance task followed by a one-way escape task in one experimental session. The apparatus was named the Elevated T-maze (ETM), which is derived from the elevated plus-maze, a widely used animal model for studying anxiety [20]. Depending on the task, the ETM generates one of the two kinds of defensive reactions, inhibitory avoidance or escape, which address two different emotions, anxiety and panic, respectively [19] [21]-[23]. Therefore, the ETM has been an important tool for the understanding of brain mechanisms underlying these two defensive responses [21] [24].

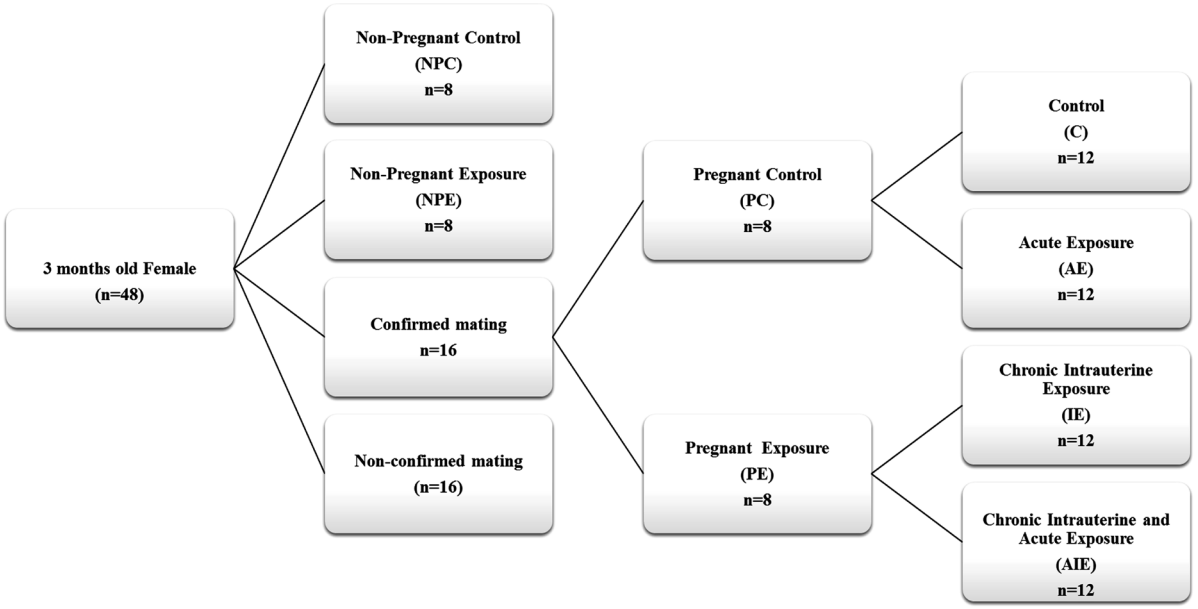
For instance, there is evidence that distinct brain structures and serotonin (5-HT) nerve fibers coming from the dorsal raphe nucleus (DRN) are involved in inhibitory avoidance and escape [25]-[27]. Specifically, these authors showed that, while serotonin released in the amygdala facilitates inhibitory avoidance, the same neuro-

transmitter inhibits escape in dorsal periaqueductal gray (dPAG). These data indicate that the neurobiological substrates, including serotonergic circuit activity, involved in these two defensive behaviors seem to be distinct. In the present study, the effects of a chronic intrauterine 22 kHz sound associated with an acute 22 kHz sound in adult offspring on serotonergic parameters were assessed in brain areas considered to have a potential role in inhibitory avoidance and escape response. These neurochemical measurements were carried out after the rats—dams and adult offspring—were submitted to the ETM paradigms used to assess anxiety and fear.

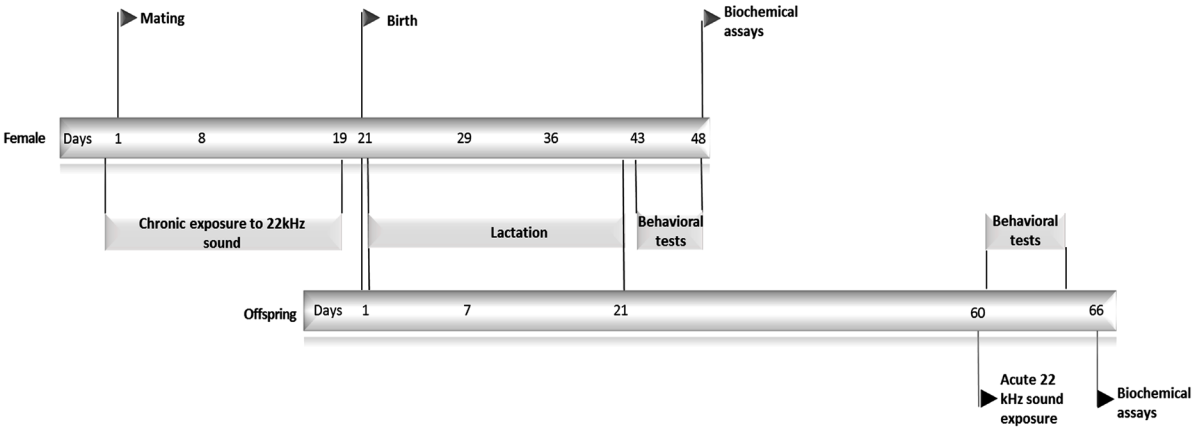
## 2. Material and Methods

### 2.1. Animals

**Figure 1** shows a schematic of the experimental groups (Panel a) and experimental design (Panel b). During experiments, all rats, dams and offspring, received chow and water *ad libitum* and were housed in an aerated room with controlled 12 h diurnal daylight/night cycles and temperature of 26˚C ± 2˚C. All experimental procedures were approved by the Ethics Committee for Animal Experimentation (CETEA/UFMG 203/2012).




(a)




(b)

**Figure 1.** Schematic showing the experimental groups (Panel a) and experimental design (Panel b).

Forty-eight 3-month-old female Wistar rats were acquired from the vivarium of the Institute of Biological Sciences, Universidade Federal de Minas Gerais. Sixteen of them were separated into two groups (n = 8, each): Non-Pregnant Control (NPC) and Non-Pregnant chronic Exposure to 22 kHz sound (NPE). According to data obtained in previous experiments performed in our laboratory [28], about 50% of females became pregnant. The remaining 32 female rats were mated, as described below, with the purpose of obtaining at least 16 pregnant rats, which were randomly assigned into two groups (n = 8, for each), Pregnant Control (PC) and Pregnant Exposure to a chronic 22 kHz sound (PE). The other sixteen female rats were removed from the experiment. The estrous cycle of females from the four groups was determined by daily vaginal smears [29], and in the proestrus stage dams from groups PC and PE were co-housed with a male. Those rats whose smears tested sperm-positive were assumed to have conceived and the developing embryos were designated to be at Day 0 of gestation.

The female pregnant rats were placed in an acoustical isolation box and exposed to a 22 kHz sound 1hour/day from the 1st to the 19th day of gestation (PE group). The rats from NPE group received the same treatment as rats from PE group, that is, they were also exposed to a 22 kHz sound during the same period. All rats from the control groups (NPC and PC) were also placed in the acoustical box, as the same period as the treated rats, except that they were not exposed to the chronic 22 kHz sound. Upon removal from the acoustical isolation box, the female rats from each of the four groups were placed in individual cages. All rats from the PC and PE groups gave birth on the 21st day.

Offspring were inspected and only male pups remained with their mothers in the proportion of 6 - 8 per dam. After the 21-day lactation period, forty-eight offspring male rats (three from each litter) were randomly divided into four groups (n = 12, each): Control pups (C), Intrauterine Chronic Exposure pups (IE), Adult Acute Exposure pups (AE), Chronic Intrauterine and Acute Adult Exposure pups (IAE). The remaining rat offspring were removed from the present experiments. The rats from the four subgroups (total n = 48) were maintained in the animal house vivarium receiving water and food *ad libitum* for two months. AE and IAE groups were placed inside the acoustical isolation box and exposed to a 22 kHz sound for 1 hour. Rats from C and IE groups were placed into the acoustical isolation box for the same period of time as rats from AE and IAE groups but were not exposed to the acute 22 kHz sound. After the acute 22 kHz treatment the rats were submitted to the ETM tasks as described below. The dams were also submitted to this behavioral test two days after the lactation period.

## 2.2. Exposure to 22 kHz Sounds

Artificial tones of 22 kHz frequency were produced using the Matlab® software [30]. A continuous 22 kHz tone [10 ms rise time, 12 s duration, 65 db sound pressure level] was emitted in sequence during one hour. Auditory stimuli were presented in an acoustical isolation box (58 × 42 × 46 cm) with a speaker taken from an electronic repellent RepemaxREP1000®, capable of emitting sounds up to 80 kHz. Each tweeter was coupled to a computer with an Asus XonarD1® model sound card. The output of sound was detected and measured using a microphone Binary Acoustic Technology model AR 125. The sounds were produced at a sampling rate of 96,000 Hz and emitted at 65 dB (adjusted using a decibel meter Polimed AM-1900).

## 2.3. Behavioral Studies

Defensive responses, inhibitory avoidance and escape related with anxiety and fear, were evaluated using the ETM behavioral model [19]. The ETM apparatus consists of three arms of equal dimension (50 × 12 cm) elevated 50 cm from the floor. One of these arms is enclosed by lateral walls (40 cm high) and stands perpendicular to the two opposite open arms. Procedure: before starting the behavioral tests an acclimation session was conducted, in which the rats were placed in and allowed to explore the open arm for 30 minutes. After 24 hours, inhibitory avoidance and escape tasks were started. Each training session included three trials. Inhibitory avoidance training session: in each of the three trials the rat was placed at the distal end of the enclosed arm facing the intersection of the arms and was allowed to explore the enclosed arm. The trial ended when the rat entered one of the open arms by placing all four paws into the open arm or remained in the enclosed arm for a maximum of 300 s. The time in which the animal remained in the enclosed arm was recorded for each trial and expressed in seconds as avoidance latency. A 30 s inter-trial interval was adopted. Escape session: Following the inhibitory avoidance task, each rat was placed at the distal end of the open arm facing the intersection of the arms. The trial ended when the rat entered inside the closed arms or remained in the open arm for a maximum of 300 s. The time in which the animal remained in the open arm was recorded for each trial and expressed in seconds as escape latency.

*Memory test*: after 72 hours of the training sessions, the rats were submitted to one more trial of inhibitory avoidance and one more trial of escape. For both tests, the ratio between the rat's performances on the last session of training and the test 72 hours later was used as an index of memory performance.

All behavioral experiments were performed in a dimly lit and quiet room between 1 pm and 5 pm. The apparatus was cleaned with 20% ethanol solution between sessions.

### 2.4. Biochemical Studies

The rats were decapitated 1 day after the end of behavioral tests. The brain was quickly removed and kept on ice. The thalamus, PFC, amygdala, PAG and hippocampus from hemispheres were immediately dissected according to the stereotaxic atlas [31]. These areas are limbic system components and play important role in modulation of emotion [32]. 5-HT and 5-HIAA contents were analyzed by high performance liquid chromatography (HPLC) [33], and the concentrations of each component were calculated by interpolation of their respective standard curves. Obtained values are expressed as ng/g of tissue. The [5-HIAA]/[5-HT] ratio was used as an index of serotonergic system activity [34].

### 2.5. Statistical Analysis

The Kolmogorov-Smirnov test was used to verify the Gaussian assumption for the variables under study at a 0.05 significance level. The distribution for all variables was normal.

For both female and offspring rats, the performance on inhibitory avoidance and escape training tasks data were analyzed using ANOVA with repeated measures in the last element (2 × 2 × 3 factorial method). For females, the factors were pregnancy, chronic exposure to 22 kHz sound and trials (time), while for rat offspring the factors were chronic intrauterine 22 kHz exposure, acute adult 22 kHz exposure and trials (time). For both female and offspring, memory performance and biochemical data were analyzed using two-way ANOVA. All values are expressed as mean ± standard error (S.E.M.). Significance level was established at 5% for all tests.

## 3. Results

### 3.1. Inhibitory Avoidance Task

#### 3.1.1. Female Performance Data

*Acquisition*: **Figure 2**, **Panel a**, shows the performance of female rats in the inhibitory avoidance training sessions. For female performance in the inhibitory avoidance task there was a significant latency decrease effect of chronic exposure to 22 kHz sound [$F_{(1,24)} = 7.13$; $P = 0.01$] and also a trial effect [$F_{(1,24)} = 13.63$, $P < 0.01$]. There were neither effects of pregnancy nor interactions between factors.

*Memory performance*: There were no significant effects of chronic 22 kHz exposure or pregnancy on female memory performance, tested in the inhibitory avoidance task, 72 hours after the training session (data not shown). There also was no interaction between these factors.

#### 3.1.2. Offspring Performance Data

*Acquisition*: **Figure 2**, **Panel b**, shows the performance of rat offspring in the inhibitory avoidance training session. Although there were no significant effects of chronic intrauterine and acute adult 22 kHz exposures, a significant effect of trials [$F_{(1,45)} = 19.36$, $P < 0.01$] and interaction between intrauterine and acute adult 22 kHz exposures [$F_{(1,45)} = 6.73$, $P = 0.01$] were observed. There were no significant interactions between the other factors (trials × chronic exposure or trials × acute exposure). The Bonferroni *post hoc* test showed significant difference between AE and IAE groups [$P = 0.02$] in inhibitory avoidance acquisition.

*Memory performance* (data not shown): There were no significant effects for chronic intrauterine or for acute adult 22 kHz exposure on offspring memory performance, tested in the inhibitory avoidance task, 72 hours after the training session. There also was no interaction between these factors.

### 3.2. Escape Task

#### 3.2.1. Female Performance Data

*Acquisition*: **Figure 3**, **Panel a**, shows the performance of female rats in the escape task during the training session.

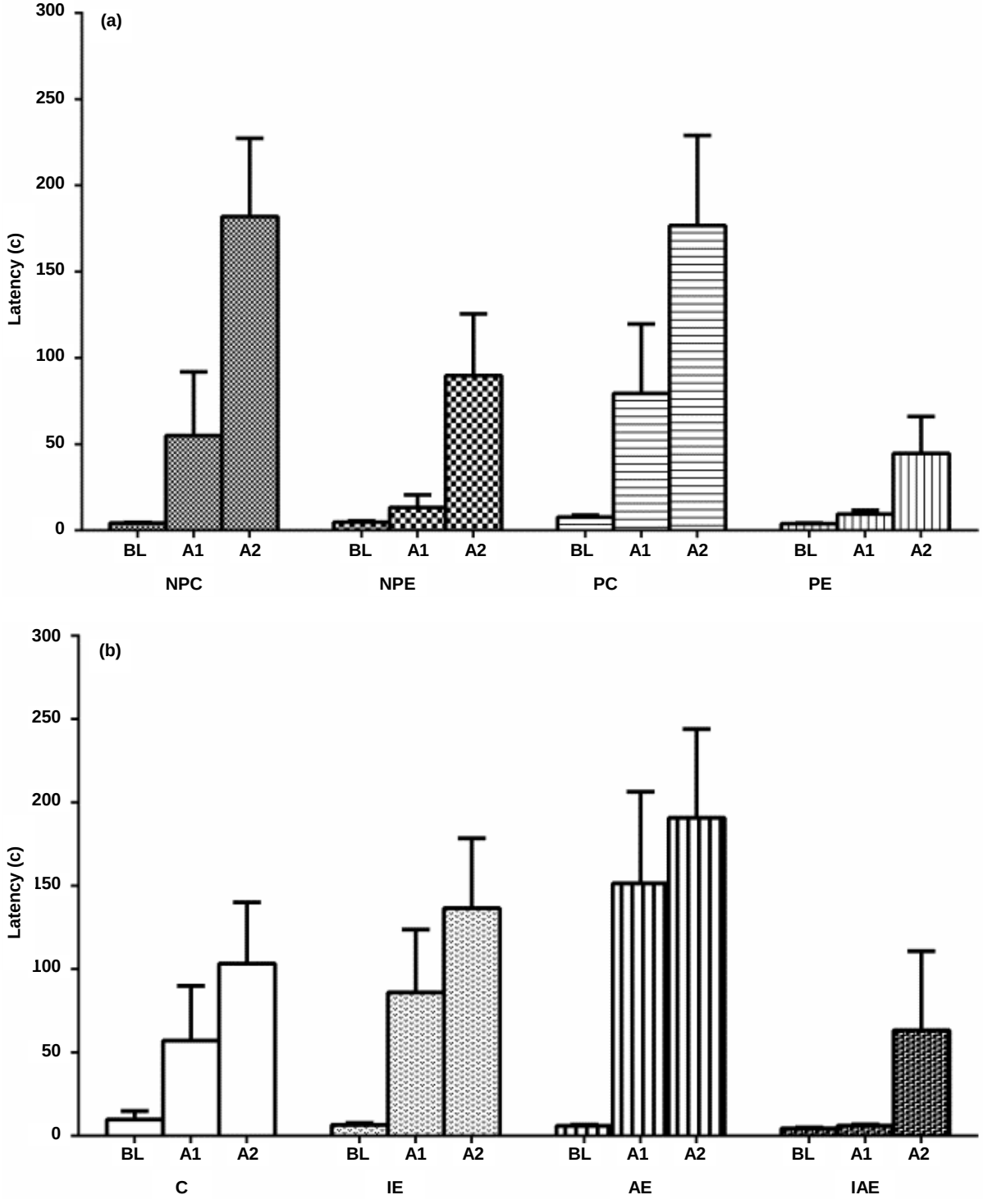



**Figure 2.** Rats performance in ETM inhibitory avoidance task expressed as latency (s). BL—Baseline; A1—Avoidance 1; A2—Avoidance 2. **Panel a**: Mean ± S.E.M. of female rat's performance. NPC—Non-Pregnant Control; NPE—Non-Pregnant Exposure to 22 kHz sound; PC—Pregnant Control; PE—Pregnant Exposure to 22 kHz sound. There were effects of trials [$F_{(1,23)} = 35.31$, $P < 0.01$] and 22 kHz exposure [$F_{(1,23)} = 7.45$; $P = 0.01$]. There was no significant effect of pregnancy or interaction between the factors. **Panel b**: Mean ± S.E.M. of adult offspring performance. C—Control; IE—Intrauterine Exposure; AE—Adult Acute Exposure; IAE—Intrauterine and Acute Adult Exposure. There were significant effects of trials [$F_{(3,46)} = 39.41$, $P < 0.01$]. There were no significant effects of intrauterine and adult exposures. There was a significant interaction between the factors [$F_{(3,46)} = 6.73$, $P = 0.01$].

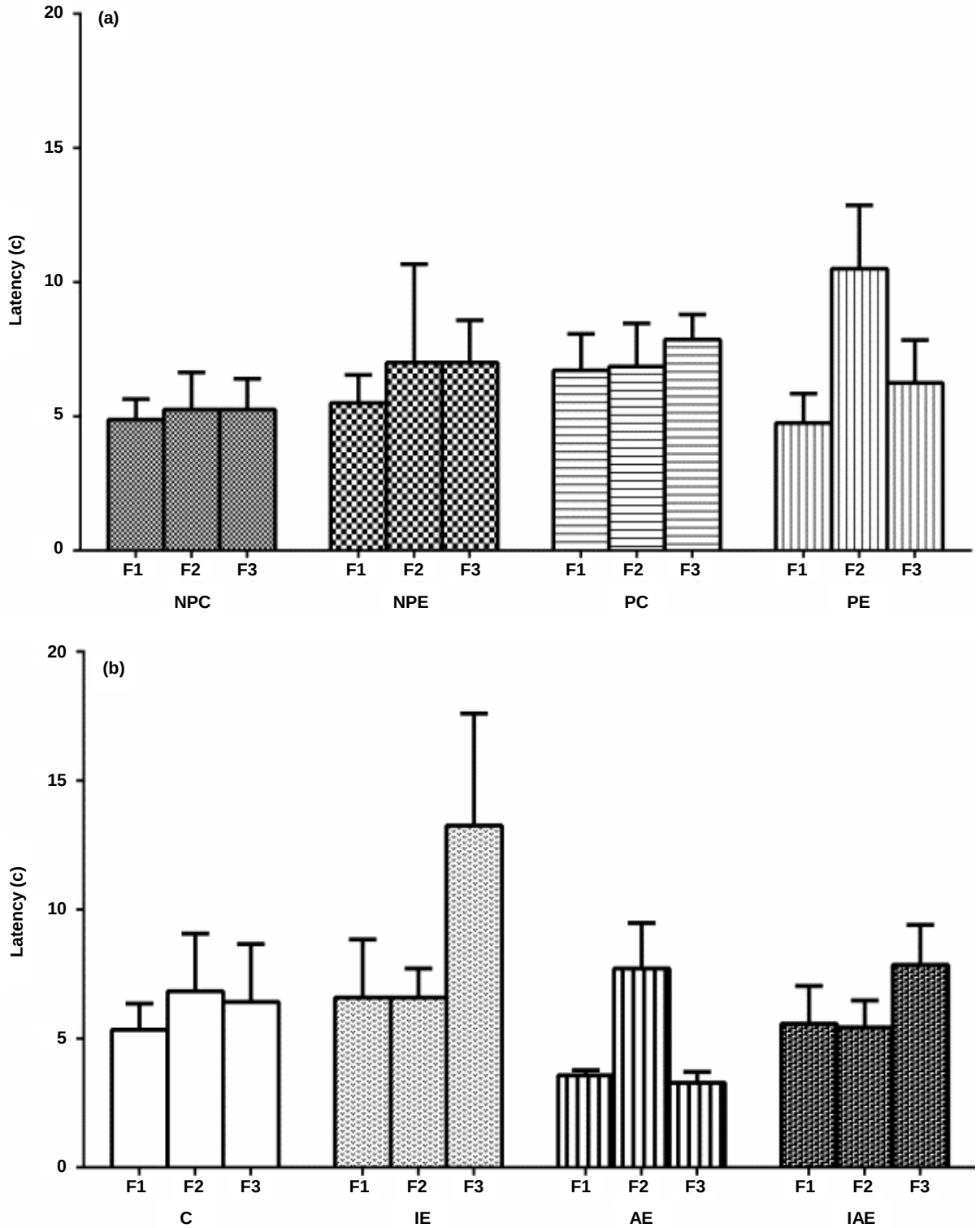



**Figure 3.** Rat's performance in ETM escape task, expressed as latency (s). T1, T2 and T3: first, second and third trials, respectively. **Panel a**: Mean ± S.E.M. of female rat's performance. NPC: Non-Pregnant Control; NPE: Non-Pregnant Exposure to 22 kHz sound; PC: Pregnant Control; PE: Pregnant Exposure to 22 kHz sound. There were no effects of trials, pregnancy, 22 kHz sound exposure and also no significant interaction between the factors. **Panel b**: Mean ± S.E.M. offspring performance. There were no effects of trials, intrauterine exposure, and adult acute exposure and also no significant interaction between the factors.

There were no significant effects of any of the treatments, chronic 22 kHz exposure, pregnancy or trials. There also were no significant interactions between the factors.

*Memory performance* (data not shown): There were no significant effects of factors (chronic 22 kHz exposure, pregnancy and trials) or interactions between) them.

#### 3.2.2. Offspring Performance Data

*Acquisition*: **Figure 3**, **Panel b**, shows the performance of rat offspring in the escape task during the training session. There was a significant effect of trials [$F_{(1,45)} = 3.22$, $P = 0.05$] and interaction between trials (time) and chronic 22 kHz exposure [$F_{(1,45)} = 5.01$, $P = 0.01$]. There were no significant effects of the other treatments (chronic or acute exposures) or interactions between them (chronic × acute exposures or trials × acute exposure).

*Memory performance* (data not shown): There were no significant effects of treatments either chronic intrauterine treatments or adult 22 kHz sound exposures on offspring memory performance tested in the escape task, 72 hours after the training session. There also was no interaction between these factors.

### 3.3. Serotonergic Parameters

**Table 1** shows biochemical data concerning 5-HT and 5-HIAA levels and serotonergic system activity ([5-HIAA]/[5-HT]) evaluated in the following rat brain areas: hippocampus, thalamus, amygdala, PAG and PFC.

#### 3.3.1. Female

Although there were no significant effects of chronic 22 kHz exposure and pregnancy on 5-HT levels in any of the five brain areas evaluated, there was a significant interaction between them in the amygdala ($F_{(3,10)} = 5.81$, $P = 0.04$). There were no significant interactions between factors on 5-HT levels in the other four regions evaluated (hippocampus, thalamus, PFC and PAG).

Neither chronic 22 kHz exposure nor pregnancy showed significant effects on 5-HIAA concentrations in any of the female brain areas analyzed. There also was no interaction between these factors.

Pregnancy had no effect on the 5-HT turnover ([5-HIAA]/[5-HT]) in any of the studied brain areas. There also was no effect of chronic 22 kHz exposure on 5-HT turnover in thalamus, amygdala and PAG. However, there were significant effects on 5-HT turnover in two areas, hippocampus [$F_{(3,11)} = 8.55$, $P = 0.01$] and PFC [$F_{(3,10)} = 6.32$, $P = 0.03$]. In addition, there were no interactions between the treatments on 5-HT turnover in any of the brain areas.

#### 3.3.2. Offspring

There were no significant effects of intrauterine exposure or acute adult exposure on 5-HT concentrations in any of the brain regions (hippocampus, thalamus, amygdala, PFC and PAD). In addition, there was no interaction between the two treatments.

Chronic intrauterine decrease and acute adult exposures increased the 5-HIAA concentrations in hippocampus. In this brain area there were significant effects of intrauterine 22 kHz exposure [$F(3,40) = 7.17$, $P = 0.01$] and acute adult exposure [$F_{(3,40)} = 5.25$, $P = 0.03$] on 5-HIAA levels.

There also were significant interactions between treatments (chronic and acute exposure) on 5-HIAA concentration in hippocampus [$F_{(3,40)} = 8.05$, $P = 0.01$] and amygdala [$F_{(3,43)} = 10.75$, $P < 0.01$]. There were no effects of factors or interactions between them in the other evaluated regions.

Chronic exposure to 22 kHz sound increase 5-HT turnover in offspring PFC [$F_{(3,42)} = 11.28$, $P < 0.01$], but there were no effects on any of the other brain areas. Acute adult exposure increase 5-HT turnover in amygdala [$F_{(3,43)} = 6.41$, $P = 0.01$]. However, there was no effect of this treatment on 5-HT turnover in any the other studied brain regions. There also was no interaction between the factors for 5-HT turnover in any of the assessed brain areas.

## 4. Discussion

The data of the present study show for the first time that the inhibitory avoidance response of female Wistar rats is affected by chronic exposure to a 22 kHz sound. Such results were not found in the escape response. In addition, this study demonstrates that chronic intrauterine exposure can also interfere with the inhibitory avoidance

**Table 1.** Serotonergic parameters.

| | [5-HT] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Female | | | | Offspring | | | |
| | NPC | NPE | PC | PE | C | IE | AE | IAE |
| Hippocampus | 171.23 ± 18.82 | 166.96 ± 9.51 | 98.25 ± 14.18 | 202.75 ± 30.60 | 81.85 ± 23.65 | 81.85 ± 23.65 | 59.76 ± 26.94 | 57.05 ± 16.13 |
| Amygdala | 180.59 ± 13.41 | 151.35 ± 19.67 | 73.28 ± 9.84 | 151.57 ± 28.60 | 293.14 ± 75.56 | 182.35 ± 29.09 | 166.14 ± 54.12 | 154.07 ± 17.17 |
| PFC | 180.59 ± 13.41 | 311.31 ± 64.60 | 101.19 ± 30.42 | 275.65 ± 59.55 | 124.08 ± 20.40 | 90.40 ± 15.57 | 103.29 ± 22.86 | 82.66 ± 7.12 |
| PAG | 944.77 ± 37.60 | 159.69 ± 83.12 | 319.88 ± 55.13 | 353.58 ± 73.42 | 617.09 ± 14.46 | 434.57 ± 54.33 | 463.38 ± 59.63 | 544.91 ± 62.37 |
| Thalamus | 729.68 ± 32.15 | 224.54 ± 0.28 | 887.63 ± 74.82 | 1443.02 ± 42.16 | 186.25 ± 26.40 | 237.96 ± 33.19 | 238.21 ± 35.06 | 284.09 ± 39.82 |
| | [5-HIAA] | | | | | | | |
| | Female | | | | Offspring | | | |
| | NPC | NPE | PC | PE | C | IE | AE | IAE |
| Hippocampus | 248.26 ± 30.61 | 182.26 ± 11.64 | 214.39 ± 7.05 | 207.68 ± 10.59 | 301.79 ± 27.23 | 293.44 ± 30.99 | 273.28 ± 28.92 | 532.13 ± 10.25 |
| Amygdala | 228.31 ± 51.53 | 128.83 ± 18.01 | 152.05 ± 14.79 | 179.95 ± 40.37 | 303.50 ± 41.38 | 194.53 ± 43.38 | 203.95 ± 15.46 | 423.68 ± 87.92 |
| PFC | 657.39 ± 62.29 | 598.35 ± 36.96 | 496.4 ± 65.18 | 976.31 ± 31.93 | 519.99 ± 43.09 | 561.14 ± 54.78 | 471.71 ± 42.57 | 618.97 ± 46.64 |
| PAG | 502.80 ± 15.85 | 658.38 ± 58.49 | 980.51 ± 23.96 | 842.13 ± 273.94 | 1403.81 ± 31.95 | 1785.30 ± 43.67 | 1311.33 ± 27.98 | 1392.11 ± 34.93 |
| Thalamus | 975.57 ± 15.41 | 1264.79 ± 22.27 | 745.03 ± 10.85 | 762.88 ± 36.53 | 706.06 ± 56.32 | 744.10 ± 54.43 | 746.06 ± 29.19 | 789.42 ± 31.54 |
| | [5-HIAA]/[5-HT] | | | | | | | |
| | Female | | | | Offspring | | | |
| | NPC | NPE | PC | PE | C | IE | AE | IAE |
| Hippocampus | 1.54 ± 0.25 | 1.09 ± 0.07 | 2.24 ± 0.40 | 1.14 ± 0.21 | 7.36 ± 1.78 | 9.00 ± 1.93 | 10.13 ± 2.48 | 11.12 ± 1.37 |
| Amygdala | 1.34 ± 0.36 | 5.24 ± 0.20 | 2.14 ± 0.49 | 1.34 ± 0.18 | 1.49 ± 0.24 | 1.25 ± 0.24 | 1.89 ± 0.38 | 3.13 ± 1.33 |
| PFC | 4.13 ± 0.66 | 2.07 ± 0.40 | 5.18 ± 0.91 | 3.46 ± 0.64 | 4.98 ± 0.51 | 7.24 ± 0.80 | 5.64 ± 0.90 | 7.70 ± 0.46 |
| PAG | 0.48 ± 0.14 | 3.01 ± 1.42 | 2.39 ± 0.22 | 2.17 ± 0.39 | 2.80 ± 0.63 | 3.27 ± 0.72 | 3.73 ± 1.31 | 2.55 ± 0.53 |
| Thalamus | 4.26 ± 1.32 | 2.07 ± 0.40 | 2.54 ± 0.02 | 1.17 ± 0.67 | 4.60 ± 0.64 | 3.67 ± 0.43 | 3.67 ± 0.51 | 3.28 ± 0.56 |

Mean ± S.E.M. (ng/g of tissue) of serotonergic parameter levels in the brain areas; PFC: Prefrontal Cortex; PAG: Periaqueductal Gray; NPC: Non-Pregnant Control; NPE: Non-Pregnant Sound Exposure; PC: Pregnant Control; PE: Pregnant Sound Exposure; C: Control; IE: Intrauterine Sound Exposure; AE: Adult Sound Exposure; IAE: Intrauterine Adult Exposure; Females: There was a significant interaction between pregnancy and chronic 22 kHz sound exposure on 5-HT concentration in amygdale [$F_{(3.10)} = 5.81$, $P = 0.05$]. There were also significant effects on 5-HT turnover in hippocampus [$F_{(3.11)} = 8.55$, $P = 0.01$] and PFC [$F_{(3.10)} = 6.32$, $P = 0.03$]. Offspring: There were significant effects of intrauterine 22 kHz exposure [$F_{(3.40)} = 7.17$, $P < 0.01$] and acute adult exposure [$F_{(3.40)} = 5.25$, $P = 0.03$] on 5-HIAA levels. There were also significant interactions between treatments on 5-HIAA concentrations in hippocampus [$F_{(3.40)} = 8.05$, $P < 0.01$] and amygdale [$F_{(3.43)} = 10.75$, $P < 0.01$]. There was a significant effect of chronic intrauterine exposure on 5-HT turnover in PFC [$F_{(3.42)} = 11.28$, $P < 0.01$]. The other factors did not have significant effects.

(but not with escape) response of offspring rats confronted with an acute 22 kHz sound when they are adults. So, the previous chronic intrauterine experience interferes with the behavioral response to an acute 22 kHz exposure later in life. There is evidence that sounds in the range of 22 kHz frequency induce rat defensive behavior [35]. However, the behavioral method used by these authors did not discriminate the two kinds of defensive responses, inhibitory avoidance and escape. These two reactions were assessed in the present study using the ETM paradigm, where they are related to inhibitory avoidance and escape, respectively [19].

The pregnant and non-pregnant female rats that were previously exposed to the chronic 22 kHz sound exhibited decreased anxious behavior when tested in the ETM apparatus (decreased inhibitory avoidance latency). On the other hand, offspring submitted to a chronic 22 kHz sound during the intrauterine period did not show any defensive response change when assessed in the ETM. However, previous chronic intrauterine 22 kHz exposures followed by an acute 22 kHz exposure during adulthood also decrease inhibitory avoidance latency in ETM task. Similarly to dam performance, offspring escape response was not changed by 22 kHz sound applied in any of the experimental conditions used in the present study. These results agree with those obtained by other authors who, using pharmacological approaches, showed that 22 kHz USV in rats is an index of anxiety but not fear [9]. These authors showed that in situations that induce anxiety, the rats sustain the 22 kHz vocalization, while fear situations produce immediate inhibition of 22 kHz vocalization. On the other hand, it appears not to agree with others, who found an anxiogenic effect of acoustic sound exposure. It is important to mention, however, that the effects of acoustic stimuli on the state of anxiety depend on different variables, such as the characteristics of the sound and the experimental animal model used. Absence of effect and both increase and decrease of state of anxiety are described in the literature. For instance, in non-pregnant females, a 65 dB white noise increase the state of anxiety [36] and no effect was shown in pregnant females subjected to a 77 dB noise of 800 Hz [37]. In addition, white noise exposure increased anxiety behavior in adult male rats [38] and decreased it in fifteen-day-old male pups [39]. Also, 20 - 45 kHz sound exposure decreased anxiety in adult male rats [40], a result that agrees with the present findings.

As the sound exposure used here was chronic, another hypothesis to be considered to explain the 22 kHz anxiolytic effects is that the dams and offspring could have learned, as a result of the repetitive exposures that the 22 kHz was not a sign of threat. This would explain the observation that over time their ETM inhibitory avoidance responses were lower compared with control rats. This hypothesis is supported by other authors who showed that repetitive sound exposure can induce habituation [41] [42]. The data obtained with offspring also agree with this hypothesis, considering that there is an interaction of effects when subjects are faced twice with a 22 kHz sound in the intrauterine and adulthood periods of development. This significant interaction could possibly be explained by the occurrence of adjustment in the neuronal net after intrauterine exposure. These potential changes induced by chronic 22 kHz sound exposure could interfere with the effects of later exposure to a similar stimulus. This hypothesis is supported by the programming neuronal plasticity theory, which states that a stimulus or an insult received in a critical period of animal development can promote anatomical, physiological, metabolic and neuropsychological adaptive changes that persist into later periods of life [12]. It is known that chronic exposure to a stressor can decrease the adjustment ability or increase the susceptibility to a subsequent stressor [43]. So, it is reasonable to consider that individuals previously exposed to 22 kHz sound in an early period of life, compared to a non-exposed individual, would be distinctly affected in terms of central nervous system and/or behavioral aspects.

Three alternative hypotheses could be proposed for explaining the neurobiological and behavioral changes caused by two sequential 22 kHz stimuli applied in different periods of offspring development: 1) a direct effect of the first exposure on the offspring neuronal net, depending on the fact that the fetus would be able to “hear” or “perceive” the environmental sound. Compared to the effects on individuals not previously exposed, a second stimulus could produce a distinct response; 2) an indirect effect of the first exposure on the offspring neuronal net, as a result of a primary effect on the dams; or 3) both effects, direct and indirect, occurring simultaneously. Thus, a distinct response to a second 22 kHz sound exposure could take place because it changes specific components of the neuronal net, which would make part of the neurobiological mechanism responsible for the behavioral response to a specific kind of environmental stimulus. In other words, a specific change caused by preceding experiences—chronic intrauterine 22 kHz sound exposure—would affect the inhibitory avoidance to a subsequent similar stimulus—acute 22 kHz—faced by the individuals in later periods of life.

With regard to the first hypothesis about a direct effect of sound on offspring, some authors stated that fetuses of sheep are able to hear environmental sounds [44] [45]. Sound is one of the first stimuli from the external environment to reach and be perceived by a nineteen-week-old human fetus [46]. However, there is evidence that rats cannot hear until two weeks after birth [47] [48]. Nevertheless, it is important to considerer the possibility of other kinds of ultrasound mechanism acting on the nervous system, which could be independent of the hearing process. Data obtained from studies using individuals of different species, including laboratory mammals, indicated that, depending on frequency and intensity, an ultrasound could cross the dam’s tissue barriers causing functional changes in fetal neural tissues [49]. Some literature supports the second hypothesis about the possibil-

ity of an indirect effect of sound, through mother, on the fetus. For instance, aversive sound caused chronic stress in pregnant women and pregnant rats [50] [51], activating the hypothalamic-pituitary-adrenal axis (HPA), increasing glucocorticoid levels in both maternal and fetal blood. It is also known that white noise increases corticosteroid levels in blood and induces hippocampal serotonergic system changes [52]. Both corticosteroids and the serotonergic system participate in cognitive and emotional outcomes [53]. Thus, it is reasonable to consider the possibility that the mechanism of action of chronic 22 kHz sound effects on offspring is direct and indirect. But as the rat offspring are not able to hear before the second week of birth, the indirect mechanism might be more probable. Further studies need to be conducted to resolve this question.

The observed effects of 22 kHz sound on ETM inhibitory avoidance and the absence of effect on escape behavior suggest that the neurobiological mechanisms related to anxiety and fear states, respectively, might be distinct. Some author shows that panic attack does not activate HPA axis [54], while the anxiety behavior involves the activation of this axis [24] [55]. As mentioned before, if an aversive sound causes chronic stress in pregnant rats [50] [51], activating the HPA axis and increasing glucocorticoid levels in both maternal and fetal blood, one can hypothesize that it could be one of the mechanisms involved in the effect of 22 kHz sound exposure on inhibitory avoidance. Further studies (e.g., glucocorticoid level determinations) need to be done to clarify this question. These data suggest that there might be a relation between 22 kHz sound and the neurobiological substrate of inhibitory avoidance but no association with escape. Likewise, the fear and anxiety behavior have different effects on the serotonergic system [56] [57].

In spite of the fact that there is evidence that serotonin uptake inhibitors decrease the number of 22 kHz ultrasonic rat vocalizations [58], we are unaware of any study about the effects of 22 kHz sound on serotonergic system activity in different brain areas. This system has an important role in modulation of anxiety state. In addition, the serotonergic activity can facilitate or attenuate the anxious behavior, depending on the brain region and the subtype of receptors involved [59]-[61].

In the present study, the following serotonergic parameter results were obtained after a chronic exposure to a 22 kHz sound during pregnancy: 1) the female and the adult offspring 5-HT concentrations were not affected in any of the assessed brain areas (PFC, PAG, amygdala, thalamus and hippocampus), 2) the adult offspring 5-HIAA concentrations were decreased in hippocampus by chronic 22 kHz sound exposure and increased when both chronic and acute stimuli were associated, 3) the serotonergic turnover rate was decreased in the female PFC and hippocampus, and increased in the offspring PFC. Regarding acute exposure (adult offspring), similar effects were observed as follows: 1) there was no effect on 5-HT levels in any of the assessed brain areas, 2) 5-HIAA concentrations were decreased in the hippocampus, 3) the serotonergic turnover rate was increased in the amygdala, an effect that was not detectable when the previous chronic and acute stimuli were associated. In short, in terms of serotonergic activity assessed by the turnover rate, these findings suggest that chronic exposure to 22 kHz sound causes a serotonergic activity decrease in the hippocampus and in the PFC of females. On the other hand, acute exposure during adulthood increases serotonergic turnover rate in the amygdala. These data indicate the involvement of limbic brain system regions in the neurobiological processes (emotional learning) affected by 22 kHz sound.

The absence of significant effects of 22 kHz sound applied during pregnancy on 5-HT levels observed in the present study accords with reports from other authors, who also did not find effects of white noise exposure on offspring hippocampal 5-HT release [62]. On the other hand, other authors showed that acute sound, including sound in the ultrasonic range, produces rapid activation of the 5-HT system in the frontal cortex, the thalamus and the hypothalamus [63]. Another hypothesis to be considered is that chronically exposed females could habituate to the stimulus. As mentioned before, this hypothesis is supported by other authors who showed that repetitive sound exposure can induce functional inactivation [42]. In addition, serotonergic parameter changes are an essential part of the adaptive process to aversive environmental stimuli [64]. This adaptive phenomenon also occurs in the amygdala, considered to be a key brain structure involved in the emotional circuit in various species [65]. Both acute and chronic stress modulate amygdala reactivity, interfering with cognitive (attention, memory) and physiological (neuroendocrine and neurochemistry) responses [66]-[68]. The plasticity in the amygdala is observed after acute and chronic stressor exposure [69]-[75].

In the present work the chronic intrauterine 22 kHz sound exposure affects female and offspring serotonergic turnover, increasing activity in the hippocampus. For female, this effect was also observed in PFC. For offspring, acute exposure increased serotonergic turnover in amygdala. The significant effects of chronic and acute treatments on 5-HIAA were observed in the hippocampus and an interaction of these treatments was found in the

hippocampus and amygdala. It is important to emphasize that there were no significant effects on serotonergic parameters in the PAG and thalamus in any of the experimental conditions used in the present study. The observation that chronic and acute 22 kHz sound exposure affect inhibitory avoidance but not escape response agrees with the findings that these acoustic sounds affect serotonergic parameters of specific brain areas, such as the hippocampus and amygdala, but not the PAG. It is known that the defensive responses, inhibitory avoidance and escape, involve distinct serotonergic pathways that originate from the raphe nucleus and continue to the forebrain and midbrain areas. The inhibitory avoidance is structured in the forebrain (e.g., amygdala, PFC), while the latter is largely integrated in the midbrain (mainly PAG) (review in [26]).

## 5. Conclusion

The present results are in agreement with the assumption that anxiety and panic are qualitatively different emotions with distinct modulator mechanisms that are related to two defensive strategies, respectively: reaction to potential threat and to proximal menace. Besides illuminating the potential of an early acoustic sound exposure for causing central serotonergic and emotional behavioral changes that can persist into later periods of life, the present data also show that the 22 kHz sound exposure represents a useful tool for understanding the mechanism of a specific defensive behavior. That is, as the 22 kHz sound exposure affects inhibitory avoidance but not escape behavior, it might be relevant for clarifying physiological and molecular aspects of emotion.

## Conflict of Interest

The authors declare no conflicts of interest.

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