LONG-TERM BIOBEHAVIORAL EFFECTS OF POSTNATAL CLOMIPRAMINE EXPOSURE ON SEROTONERGIC SYSTEMS IN A SPRAGUE-DAWLEY RAT MODEL OF OBSESSIVE-COMPULSIVE DISORDER

Undergraduate Thesis

by

Harika Kosaraju

Approved by:

Dr. Christina M. Ragan, Faculty Mentor **Date Approved: April 30, 2024**
College of Biological Sciences, Neuroscience Program
Georgia Institute of Technology

Dr. Mary Holder **Date Approved: April 30, 2024**
School of Psychology, Neuroscience Program
Georgia Institute of Technology

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ABSTRACT

Obsessive-compulsive disorder (OCD) is a psychiatric disorder characterized by difficulty controlling thoughts and impulses, and it disproportionately affects postpartum women [1]. Current treatments for OCD in adult humans include clomipramine, a tricyclic antidepressant that acts as a serotonin reuptake-inhibitor [2]. However, male rodent models of postnatal clomipramine administration show development of OCD-like behavior in adulthood [3]. Low central serotonin (5-HT) levels are associated with OCD, as 5-HT is crucial for mood stabilization [3,4]. We investigate the long-term effects of early-life anxiolytic exposure in female rats on postpartum serotonin levels. We predict that administering clomipramine to postnatal female rats causes altered maternal behavior, increased OCD-like behavior, and decreased 5-HT levels in the hippocampal brain region in postpartum adulthood, relative to controls. Decreased adulthood 5-HT levels will provide evidence that drug exposure during a sensitive developmental period results in long-term biobehavioral changes.

INTRODUCTION

Obsessive-compulsive disorder (OCD) is a psychiatric disorder characterized by difficulty controlling thoughts and impulses that affects about 2% of the population [5]. However, OCD disproportionately affects postpartum women due to the physiological and psychological stresses associated with pregnancy and childbirth, with about 11% of postpartum women affected by OCD symptoms [6]. In motherhood, OCD can be seen as excessive worries about the infant being harmed, ability to bond with the infant, and/or intrusive thoughts about harming the infant [7]. Some of the adverse effects of having postpartum OCD include exhibiting less confidence, less social support, more marital distress, and less sensitivity than mothers not experiencing postpartum OCD [7]. The negative impact of postpartum obsessive compulsive and related disorders can also affect offspring, as untreated postpartum OCD has been shown to increase the offspring's difficulties in temperament and childhood development [7]. However, treating mothers' psychiatric disorders with medication also presents concerns, as perinatal antidepressant medication can pass from mother to offspring via placental membrane or breast milk, which can also cause long-term impacts on the offspring's development and behavior [8].

OCD and mood disorders have been shown to be linked to dysregulation of various neurotransmitters, including serotonin [9]. Serotonin (5-HT) is responsible for stabilizing mood and influencing memory [3]. Because of these crucial functions, low levels of 5-HT in the brain can result in the development of psychiatric disorders. 5-HT has also been seen to impact cortical networks and influence cortical development, as it plays a role in memory consolidation, inhibition of behavior, motivation, and stress response [10]. This altered cognitive function and mood regulation is seen in psychiatric disorders such as OCD. Adult patients with disorders such as OCD and depression have been shown to also exhibit relatively decreased hippocampal volume, likely due to distress [11]. Lowered hippocampal volume is indicative of impaired hippocampal function, which may be an underlying mechanism in experiencing OCD [11]. This can lead to significant changes in 5-HT receptor expression in the hippocampus, as a majority of the 5-HT receptor subtypes are expressed in the hippocampus, including 5-$HT_{1A\text{-}B,D\text{-}F}$, 5-$HT_{2A\text{-}C}$, 5-$HT_3$, 5-$HT_4$, 5-$HT_{5A\text{-}B}$, 5-$HT_6$, and 5-$HT_7$ receptors [12].

The tricyclic antidepressant clomipramine is a commonly prescribed FDA-approved drug to treat OCD [3]. It acts by targeting serotonin and norepinephrine reuptake transporters to increase the amount of serotonin in the synaptic cleft [3]. This effect allows more serotonin to bind to receptors and affect projection areas. However, in male rats, exposure to clomipramine during the sensitive postnatal period of development during postnatal days 9-16 increases OCD-like behaviors later in life, and results in higher 5-HT2C subtype receptor gene expression in the orbital frontal cortex (OFC) of the brain [3]. The 5-HT2C receptor acts by influencing outcome prediction and decision-making through its expression in the OFC [3]. A similar behavioral and biochemical study has not been conducted in postpartum females, so this research will be working to fill the gap in the literature by studying the effects of early clomipramine exposure on maternal behaviors, OCD-like behavior, and neurotransmitter levels of rat mothers.

In our research, we conducted a novel analysis of serotonin (5-HT) in the hippocampus of Sprague-Dawley rat mothers following early postnatal clomipramine treatment compared to control mothers. **We predict that administering clomipramine to postnatal female rats causes altered maternal behavior, increased OCD-like behavior, and decreased 5-HT levels in the hippocampal brain region in postpartum adulthood, relative to controls.** Postnatal rats were given either saline or clomipramine twice daily during postnatal days 9-16. They were then raised to adulthood, mated with unrelated rats, and gave birth, after which their maternal behavior, OCD-like behavior, and hippocampal 5-HT levels were studied. Decreased adult serotonin levels provide evidence that drug exposure during a sensitive period of development results in long-term biological changes that are reflected in adult behavior and physiology.

METHODS

Subjects:

30 female Sprague-Dawley rat pups were assigned to receive intraperitoneal injections of either 0.9% saline (control) or 15 mg/kg clomipramine (experimental) twice daily during postnatal days 9-16 [3]. This time period was chosen as the rodent postnatal period of days 9-16

corresponds to the human age range of 36-40th gestational weeks to 0-2 years old, which is the time period that is crucial for maturation and growth [13]. Male rat pups were also treated and tested for OCD-like behavior, though they were not the focus of this project. The subjects were housed in a temperature-controlled room at 22℃. They were subject to a 12-hour light/dark cycle, with the light period being between 8AM-8PM and the dark period being between 8PM-8AM.

Maternal Behavior Observations:

At postnatal day 90, the female rats, previously treated with either saline or clomipramine were mated with unrelated male rats. At postpartum day 0, their offspring were evened out to include 4 males and 4 females in order to limit the confounding variables of size and sex differences between the litters. During postpartum days 1-6, mother-pup interactions and maternal behavior were observed at both 11:30 AM and 9:30 PM each day. Total licks of the pups, nursing (active crouch and passive) behavior, and self-grooming behaviors were measured.

OCD-Like Behavior Observations:

On postpartum day 7, the mother rats were tested using the hole board test. This test, a behavioral analog for compulsive behavior [14], was conducted on a 71.0 cm x 71.0 cm board raised 6 cm off the ground. The board has 16 equally and evenly spaced holes in a 4x4 grid, each 4 cm in diameter. The board is surrounded by 4 Plexiglass walls that are 44.7 cm in height. The hole board test was administered for 5 minutes, after which the total number, repetition, and location of hole pokes in this period were recorded via video camera and live observer scoring. We defined hole pokes as both the eyes and nose of the rat being submerged in the hole.

Repetitive hole pokes are indicative of the repetitive, neophobic behavior that is characteristic to OCD [15].

***Figure 1**. Hole Board Apparatus.*

A 71.0 cm x 71.0 cm hole board, raised 6 cm off the ground, was used for testing. The board contains 16 holes arranged in a 4x4 grid, all 4 cm in diameter, and is surrounded by Plexiglass walls 44.7 cm in height.

High-Performance Liquid Chromatography:

On postpartum day 21, the rat pups were weaned from their mothers and the rat dams were euthanized using CO_2. The brains of the dams were dissected, flash-frozen on dry ice, and stored in a -80℃ freezer for analysis. Brains were sectioned using a cryostat (Epredia CryoStar NX70, Kalamazoo, MI) into 50 micrometer sections at a temperature of -15°C. Tissue punches 1 mm in diameter were taken from the hippocampal region of each of the obtained brain sections using a micropuncher (Fine Science Tools, Foster City, CA). Per *The Rat Brain in Stereotaxic Coordinates* (3rd ed.), the corresponding sections for the hippocampus in rat brains are sections 26-36, with stereotaxic coordinates beginning at 6.88 mm interaural and -2.12 mm bregma and ending at 4.48 mm interaural and -4.52 mm bregma [16]. The tissue punch samples were homogenized using a glass stir rod and preserved with 200 µL of 0.07M perchloric acid for storage. High-performance liquid chromatography (HPLC), a chemical technique that separates compounds in a mixture based on hydrophobicity, was conducted to assess the amount of serotonin (5-HT) levels in the samples. The samples were pooled based upon the treatment group. They were first centrifuged in the basket centrifuge at 2500 rpm for 10 minutes, after which supernatant was collected and aliquoted into 1.7 mL microcentrifuge tubes. These microcentrifuge tubes were then spun using the microcentrifuge at 17000 rpm for 10 minutes, after which supernatant was collected and separated into multiple aliquots for HPLC analysis, each containing 230 μL of supernatant. Reverse HPLC was employed by injecting 20 microliters of the soluble material into a C18 column (Phenomenex), utilizing a nonpolar stationary phase and a polar stationary phase and a constant flow rate of 1 mL/min. 5-HT absorbance was detected at 276 nm.

Statistical Analysis:

Statistical analysis of maternal and OCD-like behaviors were conducted using independent sample t-tests to compare the results of the experimental and control groups. These t-tests were evaluated at $p < 0.05$. The percent differences in concentration of 5-HT levels between the control and experimental sample groups, obtained from the results of HPLC analysis, were compared using standard addition and qualitative analysis. In standard addition [17], various amounts of known 5-HT concentration in a standard solution (0, 2.5, and 5 μg/mL, respectively) were added to a 15 mL conical tube along with a sample aliquot each. Via LabSolutions data analysis software (Shimadzu), the resulting visualized peak indicative of 5-HT was integrated to find the area under the curve (AUC). The AUC was plotted versus the amount of standard solution added and the resulting graph was converted to a linear plot, in which the absolute value of the X-intercept was representative of the amount of 5-HT in the sample. This amount of 5-HT was divided by the weight of the tissue in the initial sample to find the amount of 5-HT (in ng) per mg of hippocampal brain tissue. For qualitative analysis, the percent difference in the amount of 5-HT in the experimental versus control samples was compared.

RESULTS

Maternal Behavior Results:

No statistically significant difference in the overall amount of time spent in active, crouched nursing posture was seen between the clomipramine-exposed and saline-exposed dams (t (27) = 0.45 , p = 0.66) (Fig. 2). However, the sharp decrease in active, crouched nursing posture on PPD Day 4 in the clomipramine-exposed dams was found to be a statistically significant difference compared to the saline-exposed dams (t (26) = 2.14, p = 0.04).

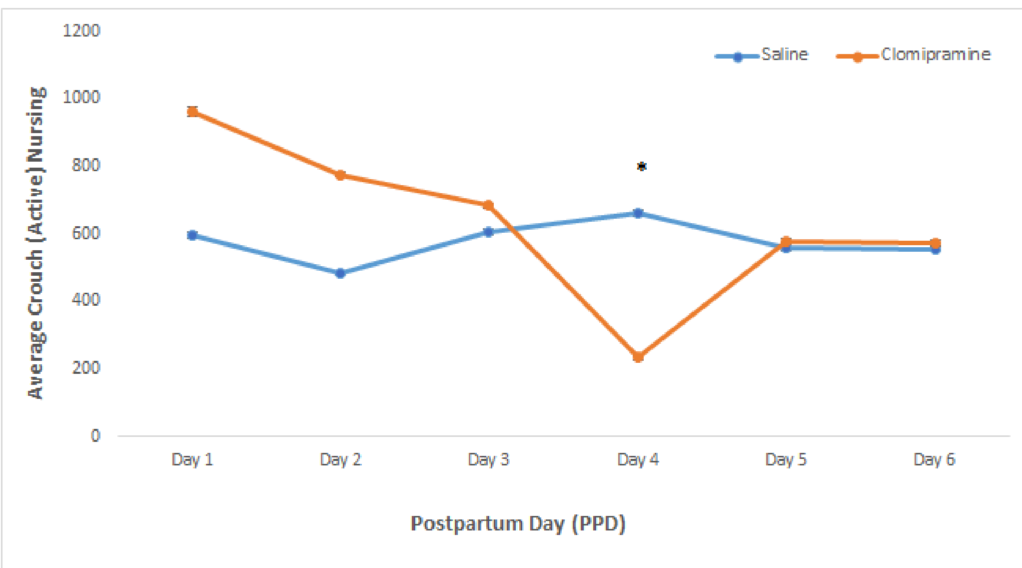

***Figure 2**. Effect of Postnatal Clomipramine/Saline Treatment on Postpartum Active, Crouched Nursing Posture in Rat Dams.*

Independent sample t-test results yield no statistically significant difference overall in total time spent in active, crouched nursing posture (t (27) = 0.45 , p = 0.66). There was, however, a statistically significant difference on PPD Day 4 between the clomipramine-exposed dams and the saline-exposed dams (t (26) = 2.14, p = 0.04). (* = $p < 0.05$).

No statistically significant difference was seen in the amount of time spent licking their pups between clomipramine-exposed dams and saline-exposed dams (t (28) = 0.06 , p = 0.95). There was also no statistically significant difference seen in the amount of time dams spent self-grooming between the clomipramine-treated group and the saline-treated group (t (28) = 0.88 , p = 0.38).

OCD-Like Behavior Results:

A statistically significant difference was seen in the average total number of hole pokes between the clomipramine-exposed dams and saline-exposed dams (t (27)= 2.69, $p < .01$) (Fig. 3A). The number of repeat hole pokes was greater in the clomipramine-exposed mothers compared to the saline-exposed mothers (Fig. 3B). However, no statistically significant difference was seen in the average number of repeat hole pokes between the clomipramine-exposed dams and saline-exposed dams (t (28)= 1.58, $p < 0.13$) (Fig. 3B).

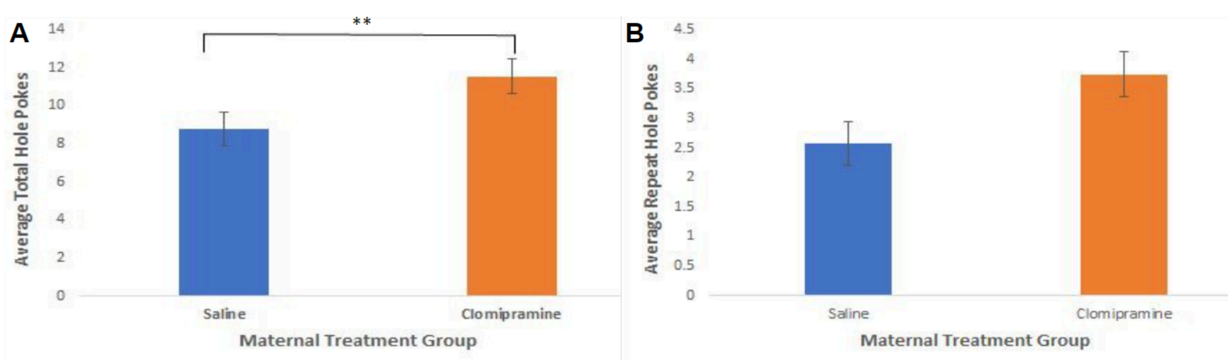

Figure 3. *Average Repeated and Total Hole Pokes in Hole-Board Test for Both Maternal Treatment Groups.*

A. Mothers exposed to early-life clomipramine made significantly more hole pokes in the hole board test ($t(27) = 2.69$, $p < .01$) and B. tended to make more repeated pokes ($t(28) = 1.58$, $p < 0.13$) than early-life, saline-treated mothers. Error bars indicate +/- SEM. (** = $p < 0.01$).

High-Performance Liquid Chromatography Results

HPLC analysis results revealed that the rats exposed to clomipramine postnatally had decreased serotonin (5-HT) levels in the hippocampus relative to the control rats that were postnatally exposed to saline (Figure 3). The saline-exposed group of rat dams exhibited 33.89 ng 5-HT per mg of brain tissue, while the clomipramine-exposed group of rat dams exhibited 16.58 ng 5-HT per mg of brain tissue. Thus, the rodents treated with clomipramine exhibited a 51% decrease in the amount of serotonin in their hippocampal region compared to the rodents treated with saline.

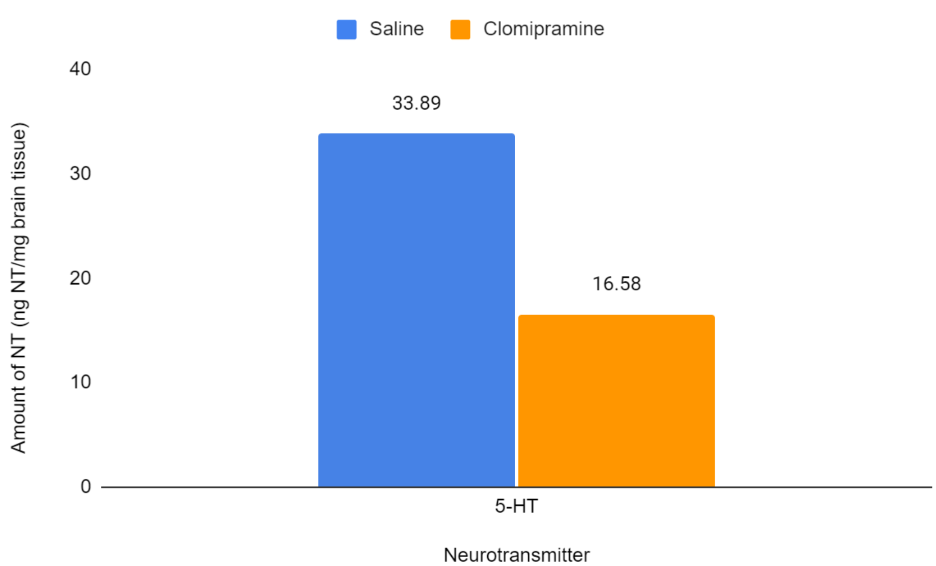

Figure 4. *5-HT Levels in the Hippocampus of Both Maternal Treatment Groups.*

Rat mothers exposed to early-life clomipramine exhibited markedly less 5-HT in their hippocampal regions relative to the saline-exposed rat mothers, seen through the amount of 5-HT in ng per mg of brain tissue. A 51.07% decrease in 5-HT between the saline-exposed and clomipramine-exposed groups was exhibited.

DISCUSSION

Results supported the hypothesis that postnatal administration of clomipramine results in altered maternal and increased OCD-like behaviors. The clomipramine-exposed group exhibited a decreased average amount of time spent in average crouched nursing posture compared to the saline-exposed group, as seen in Figure 2. This lessened active nursing time can result in decreased bonding between mother and offspring, as well as less-than-adequate growth of offspring [6]. The variability in maternal care thus supports the hypothesis that postnatal clomipramine exposure results in altered maternal behavior. Specifically, the sharp decrease in active, crouched nursing posture on PPD Day 4 in the clomipramine-exposed dams relative to the saline-exposed dams is likely indicative of inconsistent maternal behavior. This inconsistency may be due to the effect of gestational stress (GS), as GS has been shown to cause decreased arched-back nursing during the postpartum period [18]. Past studies have shown that stressed dams exhibit a 40% decrease in arched-back nursing posture by day 10 after giving birth compared to controls [18]. The exposure to clomipramine may have manifested as gestational stress due to the medication's mechanism of blocking reuptake of vital neurotransmitters that are integral to mediating the body's stress response. There was no significant difference in licking and grooming behaviors between the two groups, which may point to the notion that these behavioral measures may not be as effective in assessing the effects of medication exposure on maternal behavior. Reviews of past studies have found that 48.7% of experiments analyzing dam licking and grooming found either no change or a decrease in the behavior [19], further supporting that licking and grooming may not be a consistent enough measure to study. However, studies assessing maternal care using the chronic social stress (CSS) paradigm have found that delay in lactation is relatively increased and pup grooming time is relatively decreased in dams exposed to CSS [20], suggesting that repeated study might be required for more accurate analysis of the effect of clomipramine on maternal care and behavior.

The clomipramine-exposed group exhibited an increase in average repeated and total hole pokes in the hole-board test compared to the saline-exposed group, as seen in Figures 3A-B. Hole pokes are indicative of compulsive behavior as they are representative of a fearful and repeated escape response/aversion to new environments [21]. Thus, increased repetition and frequency of hole pokes is representative of the increased compulsions seen in OCD. The increased number and repetition of hole pokes for the experimental group thus supports the hypothesis that postnatal clomipramine exposure increases OCD-like behavior in adulthood [3]. One major limitation of the behavioral measures in this study is the use of behavioral paradigms in experimentation. While the rodent brain serves as a valid model for psychiatric disorders in humans as rats and humans both have evolutionarily similar pathophysiologies for emotional disorders [22], there are difficulties in exactly replicating neuropsychiatric conditions due to their complexities. Specifically, the additional neurocognitive deficits that are characteristic of OCD, such as executive dysfunction and decreased nonverbal memory [23], are difficult to model and assess in non-human animals. Rodent models are also subject to confounding variables such as differences in lifespan relative to humans, the effect of stress, and bodily anatomy, which can affect experimental data. Because of this variability, behavioral paradigms for OCD that exemplify strong construct and predictive validity of repetitive and compulsive behaviors may serve as more accurate measures of study, such as marble burying and nestlet shredding [24].

HPLC results also supported the hypothesis that postnatal administration of clomipramine results in decreased serotonin (5-HT) levels in the hippocampus of their brain, as seen in Figure 4, which indicates that postnatal clomipramine results in long-term serotonergic effects. As serotonin levels are implicated in OCD through serotonin's role in regulating mood and cognitive ability [3], clomipramine causing decreased adulthood serotonin levels can increase OCD-like behavior later in life. This decrease in adulthood 5-HT is likely due to downregulation of 5-HT receptors in the hippocampal region over time, as chronic exposure leads to decreased receptor responsiveness [25]. Specifically, 5-HT1A receptors have been shown to be downregulated due to selective serotonin reuptake inhibitor (SSRI) exposure [26]. Additionally, chronic exposure to sertraline, an antidepressant also used to treat OCD, has been shown to decrease 5-HT transporter binding sites in the hippocampus [27]. As clomipramine works by blocking reuptake of 5-HT in the synaptic cleft, 5-HT receptor and transporter downregulation may result in the long-term, causing this decrease in adulthood 5-HT levels. A limitation of the biological measure in this study is that there was a narrow focus on solely serotonin as the neurotransmitter of interest. Other neurotransmitters are implicated in OCD, such as glutamate and GABA [9]. These neurotransmitter systems may also be influenced due to postnatal clomipramine exposure, so future studies should focus on the wider biological mechanisms of OCD to better understand the extent to which clomipramine results in long-term biological and behavioral changes. Additionally, the subjects of the study had their behavior recorded and brains removed multiple years prior to the biochemical analyses, which may have affected experimental results due to potential tissue degradation.

This study is a novel exhibition of OCD-like behavior in a female postpartum population. It presents a better understanding of the long-term outcomes of clomipramine, along with the impact of the OCD medication on maternal mental health after pregnancy and during parenting. Mothers are an important population to study the effects of medications on and in general scientific research because of the unique experiences they present. Historically and currently, females are often excluded in biomedical research, and the female subjects that are used in rare cases are typically young and nonparous. This restriction results in a significant disparity in studying pregnant and postpartum populations. Examining maternal and OCD-like behavior will increase understanding of how OCD medication affects mothers' brain systems and influences their ability to raise children effectively, impacting both the mother and the offspring's well-being. The results of this study may serve as reason for concern in prescribing clomipramine to pregnant mothers, as it is imperative to find a treatment solution that will maintain the mother's quality and life and mental health, while also physically protecting their offspring. These long-term consequences may instead point towards utilizing other more selective antidepressant medications as a safer alternative during the perinatal/postnatal period.

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