EFFECTS OF PERINATAL SSRI EXPOSURE ON SOCIAL BEHAVIOR
AND HIPPOCAMPAL PLASTICITY IN JUVENILE RAT OFFSPRING

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by
Mariah F. Hazlett
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This thesis has been approved by
The Honors Tutorial College and the Department of Biological Sciences

__________________________
Dr. Jodi Pawluski
Research Associate, University of Rennes 1
Thesis Advisor

__________________________
Dr. Janet Duerr
Honors Tutorial College, Director of Studies
Neuroscience

__________________________
Dr. Jeremy Webster
Dean, Honors Tutorial College
Abstract

Selective serotonin reuptake inhibitor (SSRI) medications are the treatment of choice for maternal mood disorders, such as anxiety and depression, during pregnancy and the postpartum period. However, concern has been raised about the safety of these medications for the developing neonate given that SSRIs cross the placental barrier and are often found in breast milk. Untreated maternal depression alone can increase the risk for a number of negative outcomes for the mother and developing child. Therefore a thorough investigation into the effects of both maternal mood disorders and SSRI exposure on development is needed. Recent clinical work suggests that prenatal exposure to SSRI medications can increase the chance of poor social interactions in young children. However, much more work is needed in this area to understand the neurobehavioral changes underlying perinatal SSRI exposure effects on social interactions. To this end, this work focuses on how perinatal SSRI exposure affects anxiety-like and social behavior as well as hippocampal synaptic plasticity in juvenile rat offspring, while modeling aspects of maternal depression such as maternal stress. Key findings include that pregestational maternal stress and fluoxetine exposure increase different aspects of social contact behaviors in female offspring, while fluoxetine exposure decreases social grooming and increases aspects of social play behavior in male offspring. In addition, fluoxetine increases markers of synaptic plasticity in the female hippocampus while pregestational maternal stress decreases these markers in males. Together, these results expand previous work, and help to characterize the interactions between these exposures and offspring sex.
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Introduction

Maternal Depression

According to the National Institute of Mental Health (NIMH 2015), depression affects 6.7% of adults in the U.S. each year. However, it is much more common in women during pregnancy and the postpartum period, with a prevalence of up to 20% (Leung and Kaplan, 2009). Nearly 40% of women who experience postpartum depression actually develop their symptoms during pregnancy (Johnson 1997). Depression may be characterized by a combination of persistent sad or negative affect; feelings of hopelessness, worthlessness, and guilt; irritability and restlessness; loss of pleasure in activities; fatigue; cognitive impairment; changes in sleep and appetite; suicidal thoughts or actions; and physical discomforts such as aches, pains, cramps or digestive problems (NIMH 2015). Comorbid mental illnesses can occur, such as anxiety disorders and alcohol or substance abuse or dependency; if left untreated, they can worsen or otherwise complicate depression (NIMH 2015).

Untreated antenatal depression—along with maternal stress or anxiety—not only negatively affects the mother, but also has negative effects on developing offspring. Maternal depression has been associated with higher rates of pre-eclampsia, premature delivery, and neonatal complications along with impaired fetoplacental function and decreased fetal growth (Olivier et al., 2013). It is also associated with higher rates of alcohol and drug use, smoking, obesity, and poor nutrition in the mother, all of which can have negative effects on the offspring in the womb, during the early postnatal period (Olivier et al., 2013), and later in life. For example,
antenatal depression has been associated with developmental delays in offspring at 18 months, attention problems at 3-4 years, behavioral and emotional problems at 4 years, and increased anxiety at 6-9 years (Olivier et al., 2013). By adolescence, 42% of children exposed to antenatal depression display emotional disorders, though this association was only significant in adolescent girls (Olivier et al., 2013). Thus, in the interest of the health of the mother and her child, it is important for antenatal depression be treated.

**SSRI Treatment for Depression**

There are a variety of treatment options available for depression; typically, the most effective treatment is a combination of medication and therapy. The three main classes of antidepressant medications are monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants, and selective serotonin reuptake inhibitors (SSRIs). SSRIs are by far the most popular antidepressant medications (Fleschler and Peskin, 2008) due to their less severe side effects and lack of a long list of foods and drugs to avoid, a common problem with MAOIs. The most commonly prescribed SSRIs include citalopram, escitalopram, fluoxetine, paroxetine, and sertraline (NIMH 2015). SSRIs primarily act by blocking the serotonin transporter so that serotonin (5-HT) remains active in the synapse rather than being transported back into the neuron (Fig. 1). SSRIs do not act to alleviate the symptoms of depression only by increasing the level of 5-HT at the synapse; if that were the case, effects could be seen immediately following treatment. However, it often takes 2 to 4 weeks of continuous treatment for
antidepressant effects to appear. This suggests that SSRIs act through the activation of specific signal cascades that alter gene expression, a process which takes time. For example, SSRI treatment downregulates expression of the 5HT1A receptor, an inhibitory autoreceptor, and increases other factors such as brain-derived neurotrophic factor (BDNF) (Pinna 2015). SSRI treatment in animal models can alter synaptic plasticity in various areas of the hippocampus (Varea et al., 2007; Bessa et al., 2009) and influence rates of hippocampal neurogenesis; this has been suggested to mediate the behavioral effects of SSRIs (Santarelli et al., 2003; Dranovsky and Hen, 2006;
Wang et al., 2008; David et al., 2009). These findings suggest that the action of SSRIs may occur through one or more signaling cascades which alter gene expression and regulation to change the connectivity of cells within the hippocampus and thus behavior (reviewed in Pittenger and Duman, 2008).

SSRIs are prescribed to up to 10% of pregnant women in the United States and Canada (Oberlander et al., 2006; Cooper et al., 2007), with the most common being fluoxetine, prescribed to 1.4–2.1% of pregnant women (Kiryanova et al., 2013). Though SSRIs have been approved by the FDA for non-pregnant women and are generally considered safe for use, they cross the placenta and are present in breast milk (Kristensen et al., 1999; Berle et al., 2004; Kim et al., 2006; Berle and Spigset, 2011). This allows SSRIs to enter the system of the developing child and act to modulate the developing serotonergic system. At birth, neonatal plasma levels of fluoxetine and norfluoxetine, its active metabolite, average 65% and 72% of maternal plasma levels (Heikkinen et al., 2003). In umbilical vein whole blood samples from infants exposed to SSRIs, 5-HT levels are reduced by an average of 69%; levels of serotonin’s primary metabolite, 5-HIAA, are reduced by 18% (Laine et al., 2003). Up to 30% of neonates antenatally exposed to SSRIs show symptoms of poor adaptation to the extrauterine environment, such as respiratory distress; temperature instability; feeding difficulties and hypoglycemia; jitteriness, convulsions, and rigidity; irritability and sleep problems; and jaundice; these symptoms tend to be more common in neonates exposed to higher SSRI doses (reviewed in Olivier et al., 2013). These symptoms may be a result of SSRI withdrawal or overstimulation of the 5-HT system (Isbister et al.,
Antenatal SSRI exposure is also associated with an increased risk of congenital heart malformations and cardiac anomalies, especially when the fetus is exposed during the first trimester (reviewed in Olivier et al., 2013). As a result of these findings, questions have been raised concerning the effects of SSRI exposure on the developing child.

**Perinatal SSRI Exposure, Social Behaviors and Hippocampal Plasticity**

Clinical work has shown that exposure to SSRI during development can affect social and non-social behaviors. Oberlander et al. (2007) found that in 4-year olds, increased externalizing behaviors were associated with increased SSRI levels in cord blood and a history of neonatal withdrawal behaviors. Later, they found that prenatal exposure to SSRI medication and maternal depressed mood were associated with increased internalizing behaviors in 3-year olds, whereas externalizing behaviors were associated with current maternal mood; these behaviors were scored by the Child Behavior Checklist completed by the mother (Oberlander et al., 2010). In a population-based study of 6-year old children, an association was found between prenatal SSRI exposure and autistic traits such as stereotypic/repetitive behaviors, impaired interpersonal behaviors, and difficulty communicating (Marroun et al., 2014). These clinical studies point to an effect of prenatal SSRI exposure on social development; however, these studies are limited by difficulties in including appropriate non-treated depressed mothers as controls and the amount of time required for completion (following participants for a number of years).
Animal studies have also found effects of developmental exposure to SSRIs on social (i.e. play behavior) and non-social (i.e. anxiety) behaviors. For example, prenatal fluoxetine exposure was found to significantly decrease aspects of social play behavior in juvenile male rat offspring (Olivier et al., 2011; Simpson et al., 2011) and to increase neophobia (Simpson et al., 2011). A similar effect was observed in male rats postnatally treated with citalopram or fluoxetine, where juvenile play was reduced (Rodriguez-Porcel et al., 2011). Additionally, adult male and female rats postnatally treated with SSRIs showed reduced preference for a novel conspecific, indicating a decrease in adult social motivation (Rodriguez-Porcel et al., 2011). These results show that perinatal SSRI exposure can have significant effects on important behaviors related to social interactions.

Though SSRIs may act as anxiolytics during adulthood in the context of anxiety disorders, SSRI exposure during development may have different effects on anxiety-like behaviors in offspring. In rodents, neonatal SSRI exposure can increase activity in adult male offspring (Maciag et al., 2006b), and prenatal fluoxetine exposure can significantly increase anxiety-like behavior in adult male offspring (Olivier et al., 2011). Postnatal SSRI exposure has similar effects increasing anxiety-like behavior in adult male and female mice, but these results are not replicated by adult SSRI treatment (Ansorge et al., 2008). This shows that exposure to SSRIs during development can have long-term effects on anxiety-like behavior.

Not surprisingly, changes in social and anxiety-like behavior may be due to the alteration of serotonergic tone during development, as animal studies have shown that
perinatal SSRI exposure leads to suppressed serotonergic tone in adulthood (Maciag et al., 2006a). 5HT is heavily involved in the development of social responses, and modulation of this system during development may significantly alter social interactions later in life (Tonissaar et al., 2004; Crockett et al., 2010; Kiser et al., 2012). Hippocampal plasticity may also play an important role in modulating anxiety-like and social behaviors. The dentate gyrus (DG) of the hippocampus is one of two brain areas which have a high rate of neurogenesis throughout the lifespan in humans as well as rodents, the other being the subventricular zone (Eriksson et al., 1998; Pawluski et al., 2009). Hippocampal neurogenesis and other forms of plasticity are thought to play important roles in learning and memory, stress regulation, anxiety and social behaviors (Santarelli et al., 2003; Dranovsky and Hen, 2006; Hammels et al., 2015). The cornu ammonis 2 (CA2) region has recently been shown to play an important role in social memory, as lesions to this region in adult mice impaired their ability to recognize a conspecific (Hitti and Siegelbaum, 2014; Stevenson and Caldwell, 2014). New neurons and their methylation status in the DG also play a role in social behavior; Hammels et al. (2015) found that more mature or immature neurons strongly expressed DNA methyltransferase 3a (dnmt3a) in the DG of rats resilient to social defeat stress than those who were susceptible. Further work is needed to determine how exposure to SSRIs during development affects social behaviors, and what neurobiological mechanisms mediate these changes. In particular, any interactions between perinatal SSRI exposure and maternal stress need to be identified and investigated, as the effects seen in the clinical studies each include
maternal mood as a factor incompletely separable from SSRI exposure (Oberlander et al, 2007; Oberlander et al., 2010; Marroun et al., 2014).

**Perinatal SSRI Exposure and Models of Maternal Depression**

In general, human studies addressing the long term effects of SSRI exposure on child development are limited because of the long timelines of such studies, the number of participants needed, and the inability to randomly assign participants to all appropriate treatment groups. For ethical reasons depression rarely goes untreated and, of course, SSRIs cannot be given to women who show no signs of depression—especially if this may cause negative effects to her offspring. Though some studies have attempted to control for the lack of random assignment by including maternal mood as a covariate (i.e. Oberlander et al., 2006; Pawluski et al., 2012), the effects of SSRI use cannot be fully separated from those of the accompanying maternal depression (Olivier et al., 2013).

To address these issues, a variety of model organisms have been used, including rats, mice, sheep, and rabbits (Pawluski 2012). By far the most common model organism is the rodent. This is partly due to its low cost of care and multiple well-established models of aspects of depression—such as anhedonia (loss of pleasure or interest), changes in sleep patterns, and overall levels of anxiety and activity—and behavioral testing paradigms (Yan et al., 2010). A major advantage of rodent models is that their brains are relatively undeveloped at birth; their roughly 20-day gestation approximates trimesters one and two of human neural development, while their first
12 to 13 days of life approximate human trimester three (Romijn et al., 1991). Thus, both pre- and postnatal SSRI treatments can be used to investigate the effects of SSRI exposure at early developmental stages.

Although much of the animal work on the effects of perinatal SSRI on development does not use a model of maternal depression, there is more and more research showing the importance of looking at the effects of SSRI in a model of maternal stress (Pawluski 2012). When modeling aspects of maternal depression, most studies utilize a model of maternal adversity, where the pregnant dam is subjected to repeated stress in order to induce a depressive-like state. Maternal stress during gestation can induce depressive-like symptoms throughout the postpartum period (Smith et al., 2004; O’Mahony et al., 2006; Leuner et al., 2014). For example, Smith et al. (2004) and Leuner et al. (2014) found gestationally stressed rats spent more time immobile during a forced swim test and less time engaging maternal behaviors, while O’Mahony et al. (2006) found that gestational stress significantly increased immobility by 35-40% and increased pro-inflammatory immune responses. These studies show that a rat subjected to gestational stress yields an adequate model for post-partum depression. However, the clinical relevance of this model is limited. Because the early rat postpartum period is analogous to the human third trimester, rodent research involving postnatal exposure to SSRIs and maternal depressive-like symptoms could be applicable only to a limited population. This group excludes many women who have these experiences earlier in pregnancy, in trimesters one and two or even before becoming pregnant.
Recent work has shown that aspects of antepartum maternal depression may also be modeled. Huang et al. (2012) describe a model of chronic unpredictable stress where the dam is subjected to one of nine different stressors each day for a period of three weeks before mating, resulting in a depressive-like anhedonic state prior to breeding and during pregnancy as measured by the sucrose-preference test. We suspect this state persists during the early postpartum period, and we have collected maternal blood at weaning to assess maternal corticosterone and corticosteroid-binding globulin (CBG) levels to confirm this in another portion of the project. Thus, such a paradigm models antepartum depression, rather than postpartum depression.

There are only a handful of studies investigating the interaction of SSRI treatment and maternal adversity, mostly using gestational stress as a model of maternal depression. The first was conducted by Ishiwata et al. (2005), who reported that when paired with gestational stress, postnatal fluoxetine treatment restored the stress-induced reduction of dendritic spine density in the CA3 region of the hippocampus to control levels in juvenile (postnatal week 3) and adult (postnatal week 9) mice. They saw a similar effect on synaptic density, measured using electron microscopy, in the CA3 of adults (Ishiwata et al., 2005). Rayen et al. (2011) also found that postnatal fluoxetine exposure could reverse the effects of gestational stress on hippocampal cell proliferation and neurogenesis in adolescent male and female rat offspring, whereas fluoxetine alone and maternal stress alone both showed decreased levels of cell proliferation. During adulthood, Rayen et al. (2014) found more complex results, with prenatal stress having a stronger effect on hippocampal
plasticity in adult male offspring and maternal fluoxetine exposure having a stronger effect on hippocampal plasticity in adult female offspring. These studies demonstrate that the effects of SSRIs can be very different in the presence or absence of maternal stress, and can also depend on the age and sex of the offspring.

Markers of Synaptic Plasticity: Synaptophysin and PSD-95

As mentioned above, recent work points to a role for perinatal SSRI exposure in modifying neurogenesis and synaptic plasticity in the hippocampus. Synaptophysin is an integral membrane protein of small synaptic vesicles in presynaptic regions of neurons and neuroendocrine cells. Though its function is not well-understood, synaptophysin is detected in more than 90% of synaptic vesicles (reviewed in Glantz et al., 2007). PSD-95 is a protein characteristic of the postsynaptic density in more than 60% of asymmetric synapses (Glantz et al., 2007). PSD-95 helps to anchor and organize other proteins within the postsynaptic density such as the NMDA receptor (Glantz et al., 2007). Both of these proteins are commonly used as markers of synapse number (Glantz et al., 2007), while PSD-95 density also correlates with synaptic size and strength (Gray et al., 2006). Though each protein individually only marks a possible pre- or post-synaptic domain, both proteins together provides strong evidence for the strength of connectivity of neurons.

Behavioral Assays

There are numerous different behavioral assays used for rodent studies. The conclusions drawn from many such tests regarding human emotional states are
questionable, and the data collected from these assays are always up for interpretation due to the indirect nature of the test—we cannot ask a rodent “How are you feeling? Are you anxious or depressed?” and gain a valid response. However, they are the best methods we have of assessing the internal state of these animals. Researchers are constantly trying to develop new and more refined versions of behavioral tests for this reason, yielding the large number of tests currently used in rodent experiments.

The Open Field Test (OFT) was originally introduced by Hall in 1934, and is now one of the most popular methods of behavioral assessment in animal psychology (Prut and Belzung, 2003). It consists of placing an animal in a large open arena surrounded by walls for a period of time, which for rodents is a naturally stressful event. It is important to note that while this method is commonly used as an assessment of anxiety or anxiety-like behavior, it does not measure baseline levels of anxiety—rather, it measures differences in animals’ responses to an inherently stressful situation (Prut and Belzung, 2003). During each trial a variety of behaviors may be scored, including locomotion in the center of the field, total locomotion, grooming, frequency of rearing or leaning behaviors, time spent in the center, latency to enter the center, and the ratio of central/total locomotion (Prut and Belzung, 2003). Rats and mice generally walk close to the walls of the arena, a behavior called thigmotaxis. It is commonly believed that the rodents feel “safer” and less exposed when close to the walls of the arena. As a result, it is common for researchers to interpret an increase in central locomotion, time spent in center, and the center/total locomotion ratio, as well as a decrease in latency to enter the center, as indications of
decreased anxiety-like behavior (Prut and Belzung, 2003). However, these must be viewed in the context of other behavioral responses, such as changes in total locomotion or rearing behavior, which are more sensitive to stimulants (i.e. amphetamine) and a better indicator of overall activity—for this reason, the central/total locomotion ratio is often regarded as a better indicator of anxiety-like behavior than central locomotion or time spent in the center (Prut and Belzung, 2003).

While the OFT has been in use for eight decades, there is not an equivalent “classic” assessment for social behavior. This is partially because social behavior is so complex; some assessments may measure preference for a novel conspecific, others may assess play behavior, and still others may measure responses to social defeat or mating behavior. The most commonly used assessment of general social behavior is a pair social interaction test, where the experimental animal is allowed to interact with a novel, sex- and weight-matched conspecific in the home cage or a neutral environment. This paradigm was first used by File and Hyde (1978), who originally paired the trial with bright overhead light and proposed that the resulting decreases in social interaction levels could be used as a measure of anxiety (File and Hyde, 1978). Typical scored behaviors include sniffing, grooming, chasing, crawling over or under, nape attacks and other play-like behaviors, boxing, and wrestling (Varlinskaya and Spear, 2008). While many studies use general measures such as total time spent interacting, the individual behaviors displayed can be differentially affected by a variety of factors including age, sex, environmental novelty, and drug treatments (reviewed in Varlinskaya and Spear, 2008).
**Aims of the Present Study**

Most studies in this field have looked at the effects of developmental SSRI exposure with no or limited models of maternal depression and with little attention paid to potential sex differences in offspring. Developmental SSRI exposure affects a variety of measures including weight, activity, social interactions, and markers of hippocampal plasticity, and this SSRI exposure may interact with offspring sex and pregestational maternal stress as a model of maternal depression (reviewed in Kiryanova et al., 2013). Further work is required to better characterize these effects in a model that more closely mimics maternal depression, to further explore sex differences related to developmental SSRI exposure, and to characterize effects at different ages in order to differentiate between transient and persistent effects. Thus, this project is contributing to a larger project which includes adult offspring. The specific aims of this project are to understand how perinatal SSRI exposure and pregestational maternal stress can affect anxiety-like behavior, social interactions, and related changes in the hippocampus in juvenile male and female offspring. Our hypothesis is that exposures to perinatal fluoxetine and pregestational maternal stress will each have effects on offspring social behavior and markers of hippocampal synaptic plasticity. In particular, males are expected to be more sensitive to pregestational maternal stress, while females are expected to be more sensitive to fluoxetine exposure, as this is the trend found in the current literature.
**Experimental Design**

The goal of this project is to investigate the separate and combined effects of perinatal fluoxetine exposure and a model of aspects of antenatal maternal depression on social and anxiety behavior and hippocampal plasticity in juvenile male and female rat offspring. By pairing perinatal fluoxetine exposure with a model of maternal antenatal depression, as well as looking at both effects separately, the interaction between these two challenges can be further understood; such a study would not be possible in human subjects. We collected behavioral videos and brain tissue from offspring exposed to pregestational maternal stress and/or perinatal fluoxetine (Fig. 2), along with controls for a total of four groups (see Materials and Methods). Behavioral video records were scored, and brain tissue was sectioned, immunohistochemically stained, imaged, and analyzed.

**Figure 2.** Timeline of study design. GD, gestational day; PD, postnatal day.
Materials and Methods

Animals

Thirty-four adult female Sprague-Dawley rats (175-199 g, approximately 60 days of age) and 9 adult male Sprague-Dawley rats (275-299 g), from Harlan Laboratories Inc. (Indianapolis, Indiana), were kept under standard laboratory conditions in a 12:12-h light/dark schedule. Rats were initially housed in pairs in clear polyurethane bins with basic enrichment and ad libitum access to rat chow and tap water. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC, 14-H-011). All efforts were made to minimize the pain and stress levels experienced by the animals, as well as the number of animals used.

Prior to breeding, females were randomly assigned to stress or control groups (16 control, 18 stress); the stress group was housed individually and subjected to chronic unpredictable stress consisting of 0-2 stressors per day for 3 weeks. Stressors included restraint under bright light for 1 hour, overcrowding, overnight exposure to damp bedding, twelve-hour food deprivation, 5 minutes of forced swimming, and cage rotation for twelve hours.

One week following cessation of stress, all stress and control females were bred. For breeding, one female and one male were housed together and evidence of sperm in the vagina, accessed via a vaginal smear, identified gestation day (GD)1; following GD1, dams were housed individually. Females were approximately 90 days old at breeding, and each male was paired individually with up to 5 females during
breeding. Ten animals were not able to maintain a viable pregnancy, leaving 14 control females and 10 stress females.

**Fluoxetine administration**

On GD7 pregnant females were assigned to fluoxetine treatment or vehicle (saline) treatment so that half of the stressed and half of the control females received fluoxetine or vehicle. Because some females were not able to maintain a viable pregnancy, the final numbers were 6 vehicle and 8 fluoxetine control females, and 5 vehicle and 5 fluoxetine stress females. Fluoxetine or vehicle was administered orally via a cookie starting on GD10 and continued until weaning on postnatal day (PD) 21. The dams received a cookie twice a day injected with vehicle (saline) or 5mg/kg of fluoxetine in vehicle as previously described (Knaepen et al., 2013; Pawluski et al., 2014). Fluoxetine and its active metabolite, norfluoxetine, can cross the placenta and are present in breast milk, and can thus pass to offspring during gestation and lactation (Gentile 2004, 2005; Gentile et al., 2007; Kristensen et al., 1999); administration of fluoxetine by this method results in detectable levels of fluoxetine and norfluoxetine in serum of mother and pups (Knaepen et al., 2013).

This paradigm yielded four groups of male and female juvenile offspring used in the present study: 1. Control+Vehicle (CV; 22 animals, 12 male and 10 female), 2. Control+Fluoxetine (CF; 32 animals, 16 male and 16 female), 3. Maternal Stress+Vehicle (MSV; 20 animals, 10 male and 10 female), and 4. Maternal Stress+Fluoxetine (MSF; 20 animals, 10 male and 10 female). There were no
significant group effects on litter size or weight, and the average numbers of pups per litter were as follows (mean±SE): CV, 12.8±0.7; CF, 13.4±0.8; MSV, 13.7±0.2; MSF, 14.5±1.0.

On PD 1, litters were culled to 5 males and 5 females when possible. Offspring were housed with their mothers from birth until weaning on PD21, at which point they were housed with their same-sex littermates. On PD 26, offspring were weighed and underwent an open field test (OFT) as a measure of anxiety and locomotor activity, and on PD 27 they underwent a social interaction test. (The above portion of the project, behavioral testing, sacrifice, and tissue fixation were completed by Mary Gemmel, Eszter Császár, and Jodi Pawluski)

Open Field Test (OFT)

OFT was conducted during the lights-on portion of the light cycle, between the hours of 0900 and 1300 in a 60x60x35 cm darkened polyurethane box, and recorded by an overhead mounted digital camera. Rats were placed in the center zone and allowed to explore the apparatus for a period of fifteen minutes as previously described (Pawluski et al., 2009). Videos were scored by a blinded observer, by overlaying a 3x3 square grid over the field (Fig. 3); entries into peripheral and central zones were counted, as well as time spent in the central zone and the number of rears. Rearing behavior was defined as both forepaws leaving the ground. Increased entries and/or time in the center zone were considered indicative of decreased anxiety-like behavior. Increased rearing or total crosses were considered indicative of increased
activity and exploratory behavior (reviewed in Prut and Belzung, 2003). One male
was excluded as an outlier due to a score more than three standard deviations from the
average. This animal spent an unusually high amount of time in the center zone and
displayed otherwise abnormal behaviors; however, no abnormalities were observed in
the Social Interaction test, and so this animal was included in other analyses. (Video
scoring completed by Mariah Hazlett)

Figure 3. Diagram of the open field scoring grid.

Social Interaction Test (SI)

SI testing was conducted during the lights-on portion of the light cycle,
between the hours of 0900 and 1300 in the same apparatus as the OFT for a period of
ten minutes, and recorded by an overhead mounted digital camera. The OFT trial
served to habituate the experimental animal to the testing chamber. Each experimental
animal was paired with a sex- and weight-matched non-experimental animal. Videos were scored by a blinded observer. Scored behaviors include sniffing the partner; crawling over or under the partner; grooming the partner; pouncing, nape attack, or other play behavior; following the partner; running away from the partner; pinning the partner; and fighting. Time until first interaction, type of first interaction, total time spent in scored behaviors, and the number of bouts and time of each interaction type were recorded. One female was excluded as an outlier due to four social behavior scores exceeding three standard deviations from the mean. This animal had unusually high scores for bouts and time spent crawling over or under a partner, and bouts and time spent running away from a partner. However, no other abnormalities were observed in behavior, and so this animal was included in other analyses. In addition, 8 males and 8 females were excluded from this analysis, due to the unavailability of a same-sex partner of a similar weight (no more than 15g difference) at the time of testing. (Video scoring completed by Mariah Hazlett)

**Immunohistochemistry**

Juvenile offspring were sacrificed on PD 27-30. Their brains were rapidly dissected, with half being flash-frozen (used in other work). The second half was immersion fixed in 4% paraformaldehyde for 48 hrs, and then placed in saturated 30% sucrose solution for approximately a week. Immersion fixed brains were stored at -80°C until sectioning. 

Completed by Mariah Hazlett:
In order to understand how perinatal fluoxetine affects synaptic plasticity in the hippocampus of juvenile offspring, hippocampal slices were immunohistochemically stained for synaptophysin and PSD-95. Synaptophysin is a presynaptic marker and PSD-95 is a postsynaptic marker. For immunohistochemistry, immersion fixed brains of both sexes from each group were coronally sliced in 40 micron sections on a cryostat at -15°C (Leica Biosystems). Every twelfth slice was placed into 0.05 M Tris buffered saline (TBS)(pH 7.6) before being transferred to a glycol based antifreeze solution and stored at -20°C. For immunohistochemical staining, sections were rinsed between steps in TBS or TBS plus 0.01% Triton X-100 (TBST). Tissue used for PSD-95-immunoreactivity (-ir) underwent an additional antigen unmasking step, consisting of incubation in 10 mM sodium citrate buffer (pH 6.0) for 20 minutes at 80°C. Tissue used for both synaptophysin-ir and PSD-95-ir was incubated in 0.6% hydrogen peroxide solution, then blocked with 5% Normal Goat Serum (Lampire Biological Laboratories) in TBST for 30 minutes at room temperature followed by overnight incubation at 4°C in primary mouse anti-synaptophysin (1:200, Sigma Aldrich) or rabbit anti-PSD-95 (1:1000, Abcam) antibody in blocking solution. After rinsing, sections were incubated for 2 hours at room temperature in biotinylated goat anti-mouse (1:200, Vector Laboratories) or goat anti-rabbit (1:200, Vector Laboratories) secondary antibody in TBST. Further processing of the tissue was completed using the avidin-biotin complex (ABC Elite kit 1:1000, Vector Laboratories) in TBST for two hours and DAB + Nickel (3,3-diaminobenzidine; Vector) for 4 (synaptophysin) or 3 (PSD-95) minutes. Sections were mounted on
Superfrost Plus slides (Fisher Scientific), dried, dehydrated, and cover-slipped with Permount (Fisher Scientific).

For quantification, two dorsal sections in each of the cornu ammonis (CA)3 and dentate gyrus (DG) regions and 2-4 sections of the CA2 region of the dorsal hippocampus located between stereotaxic coordinates bregma -2.64 mm to -4.92 mm (Fig. 4) were analyzed for synaptophysin-ir or PSD-95-ir based on previous work (Pawluski et al., 2014; Rayen et al., 2015). Due to the small size of the CA2 region and the juvenile age, some animals had fewer sections containing this region. Immunoreactivity for all sections was examined by a blinded observer under 40x objective using a Nikon Microphot SA and Nikon DS-Qi1MC camera with Nikon NIS Elements F4.00 software. The software ImageJ64 (Wayne Rasband, NIH, Bethesda MD, USA) was used for quantification of optical densities of immunoreactive cells. The relative optical density was defined as the difference in optical density (grey level) after calibration between the area of interest and the background, which was an equivalent area adjacent to the area of interest with minimal staining.
Statistical Analysis

Data was analyzed using the software Statistica 12 (Dell Inc.). Analysis of variance tests (ANOVA) were conducted on behavioral measures in the OFT and SI with condition (maternal stress/control), treatment (fluoxetine/vehicle), and sex (male/female) as independent factors. Repeated measure ANOVAs were conducted on measures of synaptophysin density and PSD-95 density in the three regions of the hippocampus (CA2, CA3 and DG), again with condition, treatment, and sex as independent factors. Because of the well-known sex differences in behavioral measures, hippocampal plasticity and the numerous sex differences previously
observed after exposure to perinatal factors (Weinstock 2001, 2007, 2011; Rayen et al., 2015), analyses for each sex separately was done where appropriate. Significant interaction effects were analyzed in more detail by a Fisher LSD *post hoc* test in order to compare the individual groups. Significance was set at *p*<0.05.

**Results**

**Open Field Test**

Fluoxetine and maternal stress exposure increased peripheral and total crosses. When ANOVA was performed with treatment, condition, and sex as independent factors, significant treatment by condition effects were observed in both peripheral crosses (*F*(1, 85)=10.488, *p*=0.002; Table 1) and total crosses (*F*(1, 85)=9.878, *p*=0.002; Table 1) on the open field test. Fisher LSD *post hoc* analysis revealed that relative to the control (CV) group, peripheral crosses were significantly increased in the fluoxetine-alone (CF) group (*p*=0.018) and the maternal stress-alone (MSV) group (*p*=0.007); however, there was a significant decrease in peripheral crosses in the maternal stress fluoxetine (MSF) group (*p*=0.033) relative to the MSV group. Total crosses were also increased in the CF (*p*=0.011) and MSV groups (*p*=0.006) compared to the CV group; however, there was no significant effect with the MSF group.

The effects within the individual sexes differed substantially—in males, maternal stress alone significantly increased peripheral crosses, while in females fluoxetine alone increased a number of behaviors related to overall activity. Due to previous indications of sex differences in the response to fluoxetine and maternal
stress exposures (Rayen et al., 2011, 2013, 2014, 2015), further ANOVAs were performed on each of the sexes individually, with treatment and condition as independent factors. Male offspring displayed a significant treatment by condition effect \((F(1, 43)=4.104, p=0.049)\) with post-hoc tests revealing a significant increase in peripheral crosses in the MSV group \((p=0.034; \text{ Table 1})\) relative to the CV group. The majority of effects seen in the initial combined sex analysis appear to be due to differences within female offspring; indeed, within the females, additional significant treatment by condition effects emerged for peripheral crosses \((F(1, 42)=6.3407, p=0.016)\) and total crosses \((F(1, 42)=6.6832, p=0.013)\) with post-hoc tests revealing a significant increase in peripheral and total crosses in CF females compared to CV females \((p=0.024, p=0.017; \text{ Table 1})\). Additional significant treatment by condition effects were observed in juvenile females in number of rears \((F(1, 42)=4.1527, p=0.048)\) and central crosses \((F(1, 42)=5.0603, p=0.030)\), where post-hoc tests revealed rearing behavior and central crosses were increased in CF females relative to CV females \((p=0.048, p=0.017; \text{ Table 1})\). There were no other significant main or interaction effects on measures of the open field test.
Table 1. Mean (±SEM) of open field metrics in male and female offspring (n=10-16 per sex per group). * denotes significant difference from same-sex CV group (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Control + Vehicle</th>
<th>Control + Fluoxetine</th>
<th>Stress + Vehicle</th>
<th>Stress + Fluoxetine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rears</td>
<td>53.8±4.67</td>
<td>69.1±5.19</td>
<td>63.0±4.16</td>
<td>70.9±8.61</td>
</tr>
<tr>
<td>Time in Center (s)</td>
<td>10.1±1.65</td>
<td>8.2±2.05</td>
<td>10.3±3.27</td>
<td>15.5±4.90</td>
</tr>
<tr>
<td>Central Crosses</td>
<td>9.3±0.96</td>
<td>10.9±0.82</td>
<td>11.6±2.21</td>
<td>12.2±2.00</td>
</tr>
<tr>
<td>Peripheral Crosses</td>
<td>170.6±6.85</td>
<td>180.2±8.36</td>
<td>197.8±8.07*</td>
<td>173.3±8.62</td>
</tr>
<tr>
<td>Total Crosses</td>
<td>189.2±8.33</td>
<td>202.1±9.37</td>
<td>221.0±11.38</td>
<td>197.0±10.92</td>
</tr>
<tr>
<td>Central/Total Crosses</td>
<td>0.048±0.0036</td>
<td>0.054±0.0030</td>
<td>0.050±0.0068</td>
<td>0.060±0.0094</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rears</td>
<td>62.5±4.90</td>
<td>78.9±4.33*</td>
<td>73.6±7.97</td>
<td>65.5±7.01</td>
</tr>
<tr>
<td>Time in Center (s)</td>
<td>9.6±2.48</td>
<td>16.7±2.49</td>
<td>15.5±2.53</td>
<td>12.7±3.43</td>
</tr>
<tr>
<td>Central Crosses</td>
<td>9.1±1.91</td>
<td>14.2±1.00*</td>
<td>13.4±1.54</td>
<td>11.6±1.84</td>
</tr>
<tr>
<td>Peripheral Crosses</td>
<td>163.5±14.25</td>
<td>200.7±6.37*</td>
<td>196.2±13.79</td>
<td>173.6±15.03</td>
</tr>
<tr>
<td>Total Crosses</td>
<td>181.7±17.57</td>
<td>229.1±7.88*</td>
<td>223.0±16.20</td>
<td>196.8±17.54</td>
</tr>
<tr>
<td>Central/Total Crosses</td>
<td>0.046±0.0067</td>
<td>0.061±0.0028</td>
<td>0.059±0.0050</td>
<td>0.057±0.0067</td>
</tr>
</tbody>
</table>
Weight

On the day of open field testing females weighed less than males and animals exposed to fluoxetine (CF and MSF) weighed less than those exposed to vehicle (CV and MSV). Initial analysis showed main effects of sex \((F(1, 86)=1152.4, p<0.001)\) and treatment \((F(1, 86)=111.4, p=0.025)\). Upon further analysis separately by sex, only females showed an effect of treatment \((F(1, 42)=4.67, p=0.037; \text{Table 2})\) where fluoxetine exposure decreased female weight. No significant differences were seen within male offspring.

Table 2. Mean (±SEM) weight (g) at time of open field test in male and female offspring \((n=10-16 \text{ per sex per group})\). \(^\wedge\) denotes main effect of treatment in female offspring \((p=0.037)\).

<table>
<thead>
<tr>
<th></th>
<th>Control + Vehicle</th>
<th>Control + Fluoxetine</th>
<th>Stress + Vehicle</th>
<th>Stress + Fluoxetine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>77.1±2.08</td>
<td>75.1±1.13</td>
<td>79.2±2.07</td>
<td>76.4±1.33</td>
</tr>
<tr>
<td>Female</td>
<td>70.6±1.14</td>
<td>69.4±0.80(^\wedge)</td>
<td>71.0±0.79</td>
<td>68.1±1.01(^\wedge)</td>
</tr>
</tbody>
</table>

Social Interaction Test

Total Time in Scored Behaviors

Female offspring exposed to fluoxetine spent more time in scored behaviors than those exposed to vehicle. In the initial ANOVA including condition, treatment, and sex as independent factors, there was a significant treatment by sex interaction effect \((F(1, 69)=5.304, p=0.024)\) for the total time spent interacting with a partner. Post-hoc tests revealed that males exposed to vehicle spent more time interacting than females exposed to vehicle \((p=0.011)\). Following further analysis separately by sex,
only females showed any effects in this measure. A significant main effect of treatment showed that females exposed to fluoxetine (CF and MSF groups) spent more time interacting with a partner than those exposed to vehicle (CV and MSV groups) 

\( (F(1, 33)=4.957, p=0.033; \text{ Fig. 5b}) \).

\[ \]

#### Figure 5. Mean (±SEM) total time (s) spent in scored behaviors in a all animals and b males and females. b Female juvenile offspring exposed to fluoxetine (CF and MSF) spent more time interacting with a partner than vehicle-exposed females (CV and MSV; \( p=0.011 \)). ^ denotes main effect of treatment (\( p<0.05 \)). CV control+vehicle, CF control+fluoxetine, MSV maternal stress+vehicle, MSF maternal stress+fluoxetine (\( n=6-14 \) per sex per group).

\[ \]

**Crawling**

Offspring exposed to fluoxetine spent more time crawling over or under a partner than those exposed to vehicle. Initial analysis showed a significant treatment effect on the time spent crawling over or under a partner, where rats exposed to fluoxetine, regardless of maternal stress exposure, had significantly more bouts \( (F(1, 69)=11.2680, p=0.001; \text{ Table 3}) \) and spent significantly more time \( (F(1, 69)=11.2550, \)
analyzed separately, only females showed a significant effect for this measure with a significant main effect of treatment where female offspring exposed to fluoxetine had more bouts ($F(1, 33)=10.6767$, $p=0.003$; Table 3) and spent more time ($F(1, 33)=10.6767$, $p=0.003$; Fig. 6b) crawling over or under a partner than those exposed to vehicle, regardless of maternal stress exposure.

![Figure 6](image_url)  
**Figure 6.** Mean (±SEM) time (s) spent crawling over or under a partner in a all animals and b males and females. a In combined sexes, fluoxetine exposure increased time spent crawling over or under a partner ($p=0.001$). b Female offspring exposed to fluoxetine spent more time crawling over or under a partner ($p=0.003$). ^ denotes main effect of treatment ($p<0.05$). CV control+vehicle, CF control+fluoxetine, MSV maternal stress+vehicle, MSF maternal stress+fluoxetine ($n=6$-14 per sex per group).

**Grooming**

Offspring exposed to pregestational maternal stress spent more time grooming a partner; within individual sexes, fluoxetine-exposed males spent less time grooming and pregestational stress-exposed females spent more time grooming. Initial analysis
showed a significant sex by treatment interaction effect on the number of bouts \((F(1, 69)=4.8551, p=0.031)\) and time \((F(1, 69)=4.7206, p=0.033)\) spent grooming a partner with males exposed to vehicle having significantly more bouts than vehicle-exposed females \((p=0.011)\), fluoxetine-exposed males \((p=0.002)\), and fluoxetine-exposed females \((p=0.008)\), regardless of maternal stress exposure. Vehicle-exposed males also spent significantly more time grooming a partner than fluoxetine-exposed males \((p=0.005)\). Additionally, there was also a significant main effect of condition in both number of bouts and time spent grooming a partner, where animals exposed to maternal stress had more bouts \((F(1, 69)=4.9382, p=0.030; \text{Table 3})\) and spent more time \((F(1, 69)=4.0393 p=0.048; \text{Fig. 7a})\) grooming than controls, regardless of fluoxetine exposure.

Following further analysis on sex separately, both sexes displayed effects related to partner grooming. Males displayed a significant treatment effect where fluoxetine exposure decreased the bouts \((F(1, 36)=5.3419, p=0.027; \text{Table 3})\) and time \((F(1, 36)=4.7955 p=0.035; \text{Fig. 7b})\) grooming a partner relative to those exposed to vehicle, regardless of maternal stress exposure. Females, however, displayed a significant main effect of condition, where maternal stress exposure increased time \((F(1, 33)=5.2205, p=0.029; \text{Fig. 7b})\) spent grooming a partner relative to controls, regardless of fluoxetine exposure.
Pouncing/Nape Attack and Pinning

Male offspring exposed to fluoxetine had more bouts pouncing on or attacking the nape of their partner. In the initial analysis, pouncing or nape attack behavior was significantly affected by treatment. Fluoxetine-exposed offspring had significantly more bouts ($F(1, 69)=6.5982, p=0.012$; Table 3) and spent significantly more time ($F(1, 69)=4.8929, p=0.030$; Table 4) pouncing or attacking the partner’s nape relative to vehicle-exposed offspring, regardless of maternal stress exposure. Following further analysis by sex separately, these effects were partially mirrored in the male offspring while no similar effects were observed in the females. In males, there was a significant treatment effect showing that fluoxetine exposure increased the bouts ($F(1,
36)=4.9684, \( p=0.032 \); Table 3) of pouncing/nape attack behavior relative to vehicle-exposed males, regardless of maternal stress exposure.

Pinning behavior was also increased in fluoxetine-exposed male offspring. Pinning behavior, though much rarer than pouncing and nape attack behavior, showed a significant sex by treatment interaction effect in the initial analysis with fluoxetine-exposed males displaying more bouts \((F(1, 69)=6.6884, p=0.012)\) than vehicle-exposed males \((p=0.004)\) and fluoxetine-exposed females \((p=0.015)\), regardless of maternal stress exposure. Fluoxetine-exposed males (CF and MSF) also spent significantly more time pinning \((F(1, 69)=4.1195, p=0.046)\) than vehicle-exposed males (CV and MSV) \((p=0.028)\). When the sexes were analyzed separately, only males displayed a significant effect of treatment with fluoxetine-exposed males displaying significantly more bouts \((F(1, 36)=7.3745, p=0.010; \text{Table 3})\) and spending significantly more time \((F(1, 36)=7.3745, p=0.010; \text{Table 4})\) pinning their partner than vehicle-exposed males. There were no other significant main or interaction effects on measures of the Social Interaction test.
Table 3. Mean (±SEM) bouts of social interaction behaviors in male and female offspring (n=6-14 per sex per group). ^ denotes a main effect of treatment within sex (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Control + Vehicle</th>
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<th>Stress + Vehicle</th>
<th>Stress + Fluoxetine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sniffing</td>
<td>17.3±1.32</td>
<td>17.7±1.16</td>
<td>21.5±2.10</td>
<td>16.7±2.99</td>
</tr>
<tr>
<td>Crawling over/under</td>
<td>3.33±1.19</td>
<td>3.71±0.56</td>
<td>2.62±0.68</td>
<td>4.83±0.60</td>
</tr>
<tr>
<td>Grooming</td>
<td>10.1±1.64</td>
<td>5.57±0.83^</td>
<td>11.37±1.52</td>
<td>8.83±2.18^</td>
</tr>
<tr>
<td>Pouncing/nape attack</td>
<td>4.33±1.18</td>
<td>6.29±1.13^</td>
<td>3.62±1.48</td>
<td>8.83±3.13^</td>
</tr>
<tr>
<td>Following/chasing</td>
<td>8.00±1.14</td>
<td>7.86±0.73</td>
<td>6.87±1.04</td>
<td>10.3±1.74</td>
</tr>
<tr>
<td>Running away</td>
<td>3.67±0.86</td>
<td>3.57±0.79</td>
<td>1.87±0.35</td>
<td>2.50±0.76</td>
</tr>
<tr>
<td>Pinning</td>
<td>0.00±0.00</td>
<td>0.29±0.13^</td>
<td>0.00±0.00</td>
<td>0.33±0.21^</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sniffing</td>
<td>17.9±2.20</td>
<td>17.5±1.06</td>
<td>18.6±2.07</td>
<td>14.0±2.08</td>
</tr>
<tr>
<td>Crawling over/under</td>
<td>2.44±0.65</td>
<td>4.57±0.52^</td>
<td>3.12±0.91</td>
<td>7.67±2.33^</td>
</tr>
<tr>
<td>Grooming</td>
<td>6.89±1.07</td>
<td>6.21±1.00</td>
<td>7.50±0.78</td>
<td>9.50±1.09</td>
</tr>
<tr>
<td>Pouncing/nape attack</td>
<td>4.00±1.27</td>
<td>4.07±0.90</td>
<td>3.50±1.38</td>
<td>6.83±1.49</td>
</tr>
<tr>
<td>Following/chasing</td>
<td>8.11±1.03</td>
<td>8.29±0.68</td>
<td>9.62±1.97</td>
<td>9.33±1.05</td>
</tr>
<tr>
<td>Running away</td>
<td>1.56±0.58</td>
<td>2.64±0.68</td>
<td>1.87±0.55</td>
<td>2.50±0.92</td>
</tr>
<tr>
<td>Pinning</td>
<td>0.11±0.11</td>
<td>0.07±0.07</td>
<td>0.12±0.12</td>
<td>0.00±0.00</td>
</tr>
</tbody>
</table>
Table 4. Mean (±SEM) time (seconds) spent engaging in social interaction behaviors in male and female offspring (n=6-14 per sex per group). ^ denotes a main effect of treatment within sex (p=0.010).

<table>
<thead>
<tr>
<th></th>
<th>Control + Vehicle</th>
<th>Control + Fluoxetine</th>
<th>Stress + Vehicle</th>
<th>Stress + Fluoxetine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to first interaction</td>
<td>11.5±3.74</td>
<td>10.3±1.57</td>
<td>8.00±1.70</td>
<td>7.50±1.84</td>
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<tr>
<td>Sniffing</td>
<td>102.1±6.32</td>
<td>105.4±8.89</td>
<td>129.4±16.0</td>
<td>103.3±18.8</td>
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<tr>
<td>Pouncing/nape attack</td>
<td>33.3±9.32</td>
<td>44.3±7.24</td>
<td>30.6±12.8</td>
<td>60.0±21.3</td>
</tr>
<tr>
<td>Following/chasing</td>
<td>62.1±9.84</td>
<td>58.9±4.33</td>
<td>61.2±10.0</td>
<td>81.7±11.8</td>
</tr>
<tr>
<td>Running away</td>
<td>22.5±5.42</td>
<td>20.7±5.02</td>
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<tr>
<td>Pinning</td>
<td>0.00±0.00</td>
<td>1.43±0.63^</td>
<td>0.00±0.00</td>
<td>1.67±1.05^</td>
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<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Time to first interaction</td>
<td>8.78±2.41</td>
<td>10.9±3.12</td>
<td>12.9±2.61</td>
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<tr>
<td>Sniffing</td>
<td>106.7±13.0</td>
<td>113.6±8.19</td>
<td>110.6±16.6</td>
<td>80.8±12.6</td>
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<td>Pouncing/nape attack</td>
<td>27.8±9.09</td>
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<tr>
<td>Following/chasing</td>
<td>63.3±8.82</td>
<td>72.1±7.80</td>
<td>71.9±14.2</td>
<td>67.5±8.04</td>
</tr>
<tr>
<td>Running away</td>
<td>9.44±3.58</td>
<td>15.7±3.51</td>
<td>10.0±2.99</td>
<td>16.7±7.15</td>
</tr>
<tr>
<td>Pinning</td>
<td>1.11±1.11</td>
<td>0.71±0.71</td>
<td>0.62±0.62</td>
<td>0.00±0.00</td>
</tr>
</tbody>
</table>
Neuronal Markers in the Hippocampus

Synaptophysin, an integral membrane protein of presynaptic vesicles, was measured in the CA1, CA2, and DG of the hippocampus. PSD-95, a protein in the post-synaptic density of most asymmetric excitatory neurons, was measured in the same manner. Stained protein was quantified by measuring optical density, or grey level, relative to the background. Synaptophysin was differentially affected in the DG of male and female offspring. For synaptophysin density in the hippocampus, there was a treatment by area interaction effect ($F(2, 80)=3.9727, p=0.023$) where the DG of animals exposed to fluoxetine had a higher synaptophysin density than the DG of animals exposed to vehicle ($p=0.004$; Fig. 8a), regardless of maternal stress exposure. There were also significant main effects of condition ($F(1, 40)=5.5252, p=0.024$), where offspring exposed to maternal stress had lower synaptophysin density than controls, and of hippocampal area ($F(2, 80)=168.7325, p<0.001$; Table 5) with different areas of the hippocampus having different synaptophysin densities.

When doing the analysis per sex, male offspring showed a significant condition by area effect ($F(2, 40)=8.1147, p=0.001$) where the DG of males exposed to maternal stress (MSV and MSF) had lower synaptophysin density than the DG of their control counterparts (CV and CF) ($p<0.001$; Fig. 8b). There was also a significant main effect of condition ($F(1, 20)=8.1950, p=0.010$) where male offspring exposed to maternal stress had lower overall synaptophysin density and a significant main effect of hippocampal area ($F(2, 40)=173.9175, p<0.001$) with synaptophysin density being higher in the DG region of the hippocampus. In female offspring, there
was a significant treatment by area interaction effect ($F(2, 40)=4.0859$, $p=0.024$) where the DG of females exposed to fluoxetine (CF and MSF) had higher synaptophysin density than the DG of female offspring exposed to vehicle (CV and MSV) ($p=0.004$; Fig. 8b). There was also a significant main effect of hippocampal area ($F(2, 40)=50.9241$, $p<0.001$) in female offspring with synaptophysin density being highest in the DG. PSD-95 density differed significantly by area ($F(2, 80)=4.495$, $p=0.014$; Table 6), but showed no further significant effects.

![Figure 8](image.png)

**Figure 8.** Mean (±SEM) synaptophysin density (OD) in the DG. **a** CF and MSF animals had significantly higher DG synaptophysin density than CV and MSV animals ($p=0.023$). **b** Males exposed maternal stress had a lower DG synaptophysin density ($p=0.000028$) and females exposed to fluoxetine had a higher DG synaptophysin density ($p=0.04$). * denotes main effect of condition; ^ denotes main effect of treatment ($p \leq 0.05$). CV control+vehicle, CF control+fluoxetine, MSV maternal stress+vehicle, MSF maternal stress+fluoxetine ($n=6$ per sex per group).
Table 5. Mean (±SEM) synaptophysin density (OD) in male and female offspring \((n=6\) per sex per group). For DG values, see Figure 8.

<table>
<thead>
<tr>
<th></th>
<th>Control + Vehicle</th>
<th>Control + Fluoxetine</th>
<th>Stress + Vehicle</th>
<th>Stress + Fluoxetine</th>
</tr>
</thead>
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<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA2</td>
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<td><strong>Females</strong></td>
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<tr>
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<td>0.103±0.012</td>
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</tbody>
</table>

Table 6. Mean (±SEM) PSD-95 density (OD) in male and female offspring \((n=6\) per sex per group).

<table>
<thead>
<tr>
<th></th>
<th>Control + Vehicle</th>
<th>Control + Fluoxetine</th>
<th>Stress + Vehicle</th>
<th>Stress + Fluoxetine</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
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<tr>
<td>DG</td>
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<tr>
<td>DG</td>
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Discussion

Overview

Exposure to perinatal fluoxetine and pregestational maternal stress had a variety of effects within both juvenile male and female offspring. Each sex had a distinct pattern of significant effects in response to these exposures; in general, female offspring seemed to be more sensitive to these exposures, and responded predominantly to perinatal fluoxetine exposure. Male offspring, however, had fewer significant effects, and these effects were associated with both perinatal fluoxetine exposure and pregestational maternal stress.

Open Field Test

The open field test was used to assess anxiety-like behavior and general motor activity in juvenile offspring after exposure to maternal stress, perinatal fluoxetine or both. In juvenile male offspring it was found that maternal stress exposure alone resulted in increased peripheral crosses, potentially indicating an increase in anxiety-like behavior in this group. Overall there was not a strong influence of these early life exposures on either anxiety-like behavior or overall activity in juvenile male offspring.

In juvenile female offspring, perinatal exposure to fluoxetine alone increased a variety of measures in the open field, including central crosses, peripheral crosses, total crosses, and rearing behavior with no effect in the time spent in the central zone
or in the central/total crosses ratio. This suggests that these effects are more indicative of an increase in overall activity rather than a decrease in anxiety-like behavior in these animals. Interestingly, these effects were eliminated in the juvenile female offspring exposed to pregestational maternal stress and perinatal fluoxetine.

These findings on anxiety-like behavior and activity expand previous work in our lab using a somewhat different paradigm—gestational rather than pregestational stress, postnatal rather than perinatal SSRI exposure—which showed no differences in anxiety-like behavior, as measured on the open field test, in adolescent male (Knaepen et al., 2013) or male and female offspring (Rayen et al., 2011). This suggests that there may be a developmental trajectory with juvenile female offspring being particularly sensitive to developmental fluoxetine exposure compared to other ages. Additionally or alternatively, the timing of the SSRI exposure (perinatal versus postnatal) may have a long term effect. In line with this idea, recent work from our group shows that adult females have no changes in overall activity or anxiety-like behavior after postnatal exposure to fluoxetine or prenatal maternal stress (Boulle et al., 2016).

Changes in the influence of these early life exposures on anxiety-like behavior are also evident in male offspring. Recent work from our group has shown a different pattern in adult male offspring, where adult males exposed to both gestational stress and postnatal fluoxetine displayed decreased anxiety-like behavior (Boulle et al., 2015). Other groups have shown increases in anxiety-like behavior in adult male rats prenatally exposed to SSRIs (Olivier et al., 2011) and adult male mice postnatally
exposed to SSRIs (Ansorge et al., 2004, 2008). Taken together, these findings in female and male offspring begin to outline a complex relationship between offspring age, SSRI exposure timing (perinatal versus postnatal), and offspring sex, where female offspring may be more sensitive to earlier perinatal SSRI exposures and effects may be more transient (present in youth but not adulthood) and male offspring may be more sensitive over the long-term and display more changes in adult anxiety-like behavior in response to both maternal stress and developmental SSRI exposure.

**Weight**

Juvenile female offspring perinatally exposed to fluoxetine had a significantly lower body weight at the time of behavioral testing, while there were no differences in juvenile male offspring. Previous work in our lab found that fluoxetine exposure decreased weight gain in both male and female adolescent offspring (Rayen et al., 2011); in addition, at weaning, fluoxetine exposed animals weighed less than both control offspring and offspring exposed to both fluoxetine and prenatal stress, controlling for any effects of sex (Gemmel et al., 2015). McAllister et al. (2012) also found that perinatal fluoxetine exposure in female mice was associated with a lower body weight (this study did not include males). During adulthood there seems to be limited effect of perinatal fluoxetine exposure on body weight, as previous work in our lab has shown no effect of these exposures on adult male (Rayen et al., 2013) or female (Rayen et al., 2014) offspring. It is clear that developmental SSRI exposure
can decrease juvenile and adolescent offspring body weight, particularly in female offspring. However, these effects may be transient, being more pronounced in juvenile offspring and disappearing in adult offspring.

Various studies reviewed by Grzeskowiak et al. (2012) identify a variety of potential interactions between prenatal SSRI exposure, the development of the 5-HT system, HPA axis regulation, food intake and body composition regulation, maternal cortisol levels, and offspring sex, any or all of which may be involved in the effect of developmental SSRI exposure on juvenile body weight. The work investigating effects in female offspring is limited; as such, the outcomes in females are less clear than those observed in males (Grzeskowiak et al, 2012). There is much potential for further work in this area, investigating the effects of perinatal SSRI exposure and maternal stress, their interactions relating to the regulation of body weight, and the potential mechanisms involved in this regulation.

**Social Interaction Test**

Juvenile female offspring perinatally exposed to fluoxetine spent more time in scored social interaction behaviors and more time crawling over/under their partner; time spent grooming a partner increased with pregestational maternal stress exposure. While time spent in scored behaviors could be a measure for overall sociability, crawling and grooming behaviors are generally considered “contact behaviors” (Varlinskaya et al., 2008) and are involved in the formation of social bonds (reviewed
in Taylor et al., 2000). This finding expands previous research in adult female offspring postnatally exposed to fluoxetine, which showed an increase in social reproductive behaviors in these animals (Rayen et al., 2014). This work showed that adult females developmentally exposed to fluoxetine, regardless of maternal stress exposure, had increased interactions with a male and were more responsive to male advances. Adult male offspring had decreased social reproductive behavior in response to fluoxetine, but this was normalized in the male offspring exposed to prenatal stress (Rayen et al., 2013). Taken together, this work shows that increased serotonin signaling early in development can significantly increase and potentially feminize social behaviors. Serotonin is known to have a role in the sexual differentiation of the brain (Jarzab and Dohler, 1984; Dohler et al., 1991). Earlier research shows manipulations to serotonergic signaling in postnatal days 1-7 generally inhibit adult female sexual behavior (Jarzab and Dohler, 1984; Wilson et al., 1986; Dohler et al., 1991), while our work shows increased levels of serotonin in the synaptic cleft via SSRI exposure during postnatal days 1-21 facilitates adult female sexual behavior (Rayen et al., 2014). This difference in effect between the two time courses could be due to sex differences in 5-HT levels within the male and female brain appearing between postnatal days 11 and 16 (Jarzab and Dohler, 1984; Wilson et al., 1986). Alterations in serotonin signaling specifically during the postnatal period likely have long-term effects on female social behaviors (reproductive and peer-to-peer) as evidenced by the increase in juvenile social behaviors (present study) and increase in adult reproductive-related social behaviors previously reported. It may be
that the increase in adult female offspring social reproductive behaviors is a continuation of increased social behaviors in juvenile female offspring. Further work is needed in this area to understand how perinatal SSRI exposure affects these and other aspects of social behavior throughout development.

One system which may be mediating the sex differences in effects on social behaviors seen in the present study is the stress responsivity system (limbic-hypothalamic-pituitary-adrenal [HPA] axis). Changes in the HPA system can differentially impact female and male social behaviors. Taylor et al. (2000) outlines the evidence for a “tend-and-befriend” female response to stress rather than the more classical “fight-or-flight” response, which was mainly developed using male subjects (Taylor et al., 2000). This includes differences in hormonal signaling, such as the oxytocin response in females and males, which may mediate sex differences in behavioral responses to stressful situations (Taylor et al., 2000); indeed, increased oxytocin is associated with increases in social contact and grooming in female rats (Argiolas and Gessa, 1991; Witt et al., 1992), the same behaviors which are increased by fluoxetine and pregestational maternal stress exposures, respectively. This suggests potential effects of perinatal SSRI exposure on the stress response and HPA axis, particularly in female offspring.

Juvenile male offspring perinatally exposed to fluoxetine spent less time grooming their partner, more time pinning their partner and displayed more bouts of pinning and pouncing/nape attack behavior but did not differ from other groups in the total time spent in social interaction. Pinning and pouncing/nape attack are “play
fighting behaviors” (Varlinskaya et al., 2008) and are generally more common in males than females (Meaney and Stewart, 1979; Pellis and Pellis, 2004). Other research has found that prenatal (Olivier et al., 2011), perinatal (Simpson et al., 2011) and postnatal (Day 8–21) (Rodriguez-Porcel et al., 2011) SSRI exposure decreased social play in juvenile male rats. While prenatal exposure to fluoxetine resulted in decreased bouts of pinning behavior (Olivier et al., 2011) and later postnatal fluoxetine decreased both pinning and boxing behaviors (Rodriguez-Porcel et al., 2011), it is unclear what specific behaviors were affected by perinatal citalopram (Simpson et al., 2011). Discrepancies between findings of the present work and previous work may be due to many methodological differences between studies, including the duration of SSRI exposure (prenatal, perinatal, postnatal), the SSRI dose, administration method (via the dam or injection to the pups), and type of SSRI used. In addition, at least two of these previous papers isolated the experimental animal for a time before assessing social play behavior in order to facilitate the appearance of social behaviors (Olivier et al., 2011; Rodriguez-Porcel et al., 2011), and in the third it is unclear whether the test was performed with a littermate or a novel conspecific. Regardless of these differences, it does appear that developmental fluoxetine exposure affects social play behaviors within male offspring. Because studies investigating the effects of developmental SSRI exposure on juvenile social interaction are quite limited, more work will need to be conducted before any strong conclusions are drawn. Further studies should investigate the effects of perinatal SSRI and pregestational maternal stress exposures with similar SSRI doses and additional tests.
of social interaction such as the social approach-avoidance test and the prosocial helping behavior task (Brodkin et al., 2004; Bartal et al., 2011).

Juvenile male and female rats inherently engage in some behaviors at different frequencies, for example juvenile males engage in social play behavior more often than females (Meaney and Stewart, 1979; Pellis and Pellis, 2004) and females engage in social grooming more often than males (Meaney and Stewart, 1979). Sex differences in typical juvenile social interaction behaviors arise from prepubescent sexual differentiation of hormone signaling cascades and sexually dimorphic brain areas (Meaney 1989), as illustrated by a decrease in social play behavior in juvenile male rats resulting from prepubertal gonadectomy (Cooke and Woolley, 2009) and the defeminization of social play behavior in juvenile females perinatally exposed to bisphenol A (Porrini et al., 2005). Previous work in our lab has shown that early postnatal fluoxetine exposure decreases anogenital distance in juvenile male offspring, which is generally much larger in males than females (Rayen et al., 2013). This finding was associated with decreased area of the sexually dimorphic nucleus of the preoptic area (SDN-POA) and changes in male sexual behavior in adult offspring (Rayen et al., 2013). This shows that developmental fluoxetine exposure can indeed have an effect on the process of sexual differentiation, providing a clear mechanism through which perinatal exposures could affect social interaction behaviors differently in males and females. Given that both sexes experienced significant but very different effects on grooming behavior, it is likely that exposure to pregestational maternal stress and perinatal fluoxetine interacts with these processes of sexual differentiation.
during development, leading to changes in social behaviors in a sex-dependent manner.

**Hippocampal Synaptophysin and PSD-95**

Recent research has shown that the hippocampus can play an important role in social cognition (Rubin et al., 2014; Hitti and Siegelbaum, 2014; Stevenson and Caldwell, 2014). In the present study, juvenile female offspring perinatally exposed to fluoxetine had higher synaptophysin density in the dentate gyrus of the hippocampus. This finding expands previous work in our lab showing a developmental trajectory of perinatal SSRI effects on synaptophysin density in the hippocampus. No significant effects of SSRIs on hippocampal synaptophysin density are observed in offspring at weaning (Gemmel et al., 2015), and developmental SSRI exposure decreases synaptophysin density in the dentate gyrus of adult female offspring (Rayen et al., 2015). Thus, the specific effects of developmental SSRI exposure on synaptophysin density in the dentate gyrus of female offspring may vary with the age of the offspring, particularly in females.

Juvenile male offspring exposed to pregestational maternal stress had lower synaptophysin density in the dentate gyrus of the hippocampus. No changes in juvenile or adolescent male hippocampal synaptophysin density in response to maternal stress or fluoxetine exposure have been previously reported. As such, much further work is required in order to understand the neural mechanisms mediating this effect and any potential impacts on behavior.
The changes in female synaptophysin in the dentate gyrus parallels some changes in juvenile female social behavior, such as the increase in time spent in total behaviors and time spent crawling over or under a partner in the social interaction test. However, the changes in male synaptophysin in the dentate gyrus in response to pregestational maternal stress do not align well with changes in juvenile male social behavior, which were more sensitive to fluoxetine exposure. It is likely that changes within other brain areas, such as the amygdala and hypothalamus, may better explain these changes in male social behavior. Despite the lack of parallel effects in male behavior and hippocampal synaptophysin density, it is clear that exposure to perinatal fluoxetine and pregestational maternal stress affects this marker for neural synapses in a sex-dependent manner.

Though significant effects were observed in synaptophysin density in male and female DG in response to pregestational maternal stress and perinatal fluoxetine, respectively, no corresponding changes in PSD-95 density were observed in this or any other hippocampal areas. This may be due to the fundamental differences of synaptophysin and PSD-95 as synaptic markers. While synaptophysin is an integral vesicular membrane protein (Wiedenmann and Franke, 1985; Navone et al., 1986) and thus present in the vast majority of synapses, PSD-95 is a scaffolding protein involved in the anchoring of GluN2 subunits to the post-synaptic density, and is thus concentrated in excitatory synapses (Squire et al., 2013; reviewed in Sheng and Hoogenraad, 2007). Thus, viewing these markers only as “presynaptic” and “postsynaptic” ignores additional information conveyed by these two proteins—
instead, synaptophysin is more of a “general presynaptic” marker and PSD-95 is an “excitatory postsynaptic” marker. The sensitivity of synaptophysin over PSD-95 in this experiment could indicate that the effects are specifically observed in non-excitatory synapses, such as inhibitory or modulatory synapses, such as those where 5-HT is the main neurotransmitter. Though PSD-95 may be involved in synaptic plasticity at excitatory synapses via interactions with the NMDA receptor (Hata and Takei, 1999), inhibitory and modulatory connections have important roles in this process as well (Squire et al., 2013), and changes in 5-HT levels during the development of these systems could have long-term effects. Alternatively, exposure to perinatal fluoxetine or pregestational maternal stress may have effects on the pattern of synaptic pruning within the juvenile offspring. Glantz et al. (2007) found that in humans, levels of synaptophysin and PSD-95 both increased through childhood until early adolescence, when synaptophysin levels began to decrease and PSD-95 levels plateaued. The authors of this study suggested that excitatory synapses lacking PSD-95 may be selectively eliminated during the pruning process. If this is the case, alterations to the pruning process in the juvenile offspring in our study could lead to changes in synaptophysin density but not PSD-95 density.

Clinical Implications

While there is still much work to be done in this area, many important clinical implications are beginning to arise from animal research relating to SSRI use during
pregnancy and antenatal depression. Though it may seem odd at first to consider, it is possible that, given the striking sex differences in sensitivity to perinatal SSRI exposure or maternal stress, physicians may consider treating women differently depending on the sex of her developing child. Pregnancy is always a delicate time for balancing medications, health benefits and risks for both the mother and child. Results from these studies provide additional information regarding neurobehavioral effects in developing offspring exposed to these challenges, and may encourage physicians to try and minimize the dosage of SSRIs given to pregnant women, provided depressive symptoms due not escalate, and possibly explore additional, complimentary therapies such as cognitive behavioral therapy and moderate exercise.

**Future Directions**

There is much that is still poorly understood regarding perinatal exposure to SSRIs effects on development, particularly in combination with models of maternal depression. Further work in our lab will investigate these same measures in adult offspring, as well as other measures in both juveniles and adults, including markers of HPA-axis activity, markers of synaptic plasticity within the prefrontal cortex, and changes in other proteins which are markers for global methylation (Dnmt3a) or are involved in regulation of the HPA-axis (glucocorticoid receptors, arginine vasopressin receptor). We believe this will further illuminate the effects of these exposures on offspring, both early and later in life, in various aspects of behavior, physiology, and
neurodevelopmental outcomes. Other potential avenues of research include comparing the effects of fluoxetine with other SSRIs and at various dosages, and investigating the specific mechanisms mediating the observed sex differences in behavior, weight, and changes in plasticity.

Conclusions

Findings from the present study highlight the importance of using animal models to investigate the effects of perinatal SSRI exposure on neurobehavioral outcomes, particularly with a model of aspects of maternal depression. Key findings show long-term and sexually dichotomous effects of perinatal SSRI exposure on social behavior and hippocampal plasticity in juvenile offspring. This contributes to our understanding of how exposures to perinatal SSRI medication affect development. Understanding these effects helps to inform us of potential benefits and risks of using these medications to treat maternal mood disorders.
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