



# *Effects of maternal stress and postnatal fluoxetine exposure on affective-like behaviors and hippocampal cell proliferation in adolescent rats*

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## List of abbreviations

<b>5-HIAA</b>	5-hydroxyindoleacetic
<b>5-HT</b>	5-hydroxytryptophan/serotonin
<b>5-HTT</b>	serotonin transporter
<b>ACTH</b>	adrenocorticotrophin hormone
<b>ADHD</b>	attention-deficit disorder
<b>ANOVA</b>	analysis of variance
<b>AVP</b>	arginine-vasopressine
<b>BDNF</b>	brain-derived neurotrophic factor
<b>CORT</b>	corticosterone
<b>CF</b>	control fluoxetine
<b>CNS</b>	central nervous system
<b>CRH</b>	corticotropin-releasing hormone
<b>CV</b>	control vehicle
<b>DA</b>	dopamine
<b>DG</b>	dentate gyrus
<b>DHPG</b>	dihydroxyphenylglycol
<b>EEG</b>	electroencephalography
<b>FHRV</b>	fetal heart rate variability
<b>FST</b>	forced swim test
<b>GCL</b>	granular cell layer
<b>GD</b>	gestation day
<b>GR</b>	glucocorticoid receptor
<b>HPA</b>	hypothalamic-pituitary-adrenal
<b>IGF-1</b>	insuline-like growth factor-1
<b>IHC</b>	immunohistochemistry
<b>Ki67-ir</b>	Ki67-immunoreactive
<b>MAO</b>	monoamine-oxidase
<b>MCA</b>	middle cerebral artery
<b>MSF</b>	maternal stress + fluoxetine
<b>MSV</b>	maternal stress + vehicle
<b>NE</b>	norepinephrine
<b>OFT</b>	open field test
<b>P1</b>	postnatal day 1
<b>PBS</b>	phosphate buffered saline
<b>PPD</b>	postpartum depression
<b>PVN</b>	paraventricular nucleus
<b>S100B</b>	S100 calcium-binding protein B
<b>SGZ</b>	subgranular zone
<b>SSRI</b>	serotonin reuptake inhibitor
<b>SVZ</b>	subventricular zone
<b>TPH</b>	tryptophan hydroxylase
<b>trp</b>	tryptophan
<b>TSH</b>	thyroid-stimulating hormone

## Preface

This thesis implicates the end of my Master at the translational University Limburg. These 5 years of university were a nice experience in my life. I've got the chance to study and expand my knowledge. It prepared me for the next step in my life. Especially during my Master internship, I acquired a lot of knowledge concerning research. Therefore I want to thank some special people for their support and guidance during this period of 8 months.

First of all, I want to thank my daily supervisor dr. Jodi Pawluski. She learned me how to perform good research and think critical. She supported me with practical and theoretical aspects during this internship. Thank you so much for your help, I really appreciate what you have done for me. Next, I want to say thank you to dr. Jos Prickaerts, because he provided me a really interesting and fascinating project. I also want to thank all the technicians for helping me with experiments in the lab, with special thank to Denise Hermes, for helping with the animals, and to Hellen Steinbusch, for the lab introductions. I also want to thank my friends, Romina Gentier, Sarina Gerard, Lore Delbroek and Ingrid Meex, for the support and lunch every day.

Finally, I want to say thank you to my family, with special thank to my sister Liesbeth Rayen. They were always there for me when I needed them. I also want to thank my best friend Maarten Schuurmans, for his support all those years. This thesis will be dedicated to my both parents, Christianne Vangeloven and Peter Rayen, who are not here anymore and hadn't the chance to see me growing up. Mom and dad, I love you!

Thank you for all the support!

Ine

## **Abstract (English)**

Stress during gestation has marked effects on offspring development and results in postpartum depressive-like behavior in animal models. Postpartum depression (PPD) is a growing health problem, which affects 15% of women worldwide. Currently, selective serotonin reuptake inhibitor (SSRIs) medications are commonly used for treatment of PPD. Unfortunately there is very little research on the effect of maternal depression and perinatal SSRI exposure on adolescent offspring development. Therefore the aim of this study was to determine how postnatal maternal fluoxetine exposure affects neurobehavioral outcomes adolescent offspring exposed to maternal stress. To do this, gestationally stressed and non-stressed Sprague-Dawley rat dams were divided into 2 groups: 1) fluoxetine treated (5mg/kg/day) or 2) vehicle. On postnatal day 1 (P1) treatment was administered to mothers via minipump implants (Alzet). Offspring were weaned at P21 and four groups of male and female offspring were used: 1) maternal stress + fluoxetine, 2) maternal stress + vehicle, 3) fluoxetine alone, 4) vehicle alone. Adolescent offspring (approximately 35 days of age) were tested in the open field and the forced swim test to assess anxiety- and depressive-like behaviors, respectively. After testing, offspring were sacrificed and brains were analyzed for levels of cell proliferation (Ki67-labeling) in the dentate gyrus of the hippocampus. Results demonstrate that maternal fluoxetine increases depressive-like behavior in adolescent offspring exposed to maternal stress but has no effect in non-stressed offspring, regardless of sex and maternal behavior. In addition, postnatal fluoxetine exposure appears to increase hippocampal cell proliferation partially in adolescent offspring exposed to maternal stress, but decreases hippocampal cell proliferation in non-stressed offspring. This research provides important evidence of the effects of postnatal fluoxetine exposure on offspring development.

## **Abstract (Nederlands)**

Stress tijdens de zwangerschap heeft ernstige gevolgen voor de ontwikkeling van de nakomelingen en resulteert in postnataal depressie-gerelateerd gedrag in diermodellen. Postnatale depressie is een groeiend gezondheidsprobleem, dat 15% van de vrouwen wereldwijd aantreft. Tegenwoordig worden vooral selectieve serotonine heropname remmers (SSRIs) voorgeschreven voor de behandeling van postnatale depressie. Er is zeer weinig onderzoek naar het effect van prenatale stress en perinatale SSRI blootstelling op de ontwikkeling van adolescente nakomelingen. Het doel van de studie was te bepalen hoe postnatale blootstelling aan fluoxetine effect heeft op de ontwikkeling van adolescente nakomelingen. Sprague-Dawley rat moeders werden wel of niet blootgesteld aan prenatale stress en verdeeld over twee behandelingsgroepen: 1) fluoxetine behandeling (5mg/kg/dag) of 2) vehicle. Op postnatale dag 1 (P1) werd de behandeling toegediend via minipomp implantaten (Alzet). Het voeden van de nakomelingen door de moeders werd gestopt op P21 en vier groepen nakomelingen werden gebruikt: 1) prenatale stress + fluoxetine, 2) prenatale stress + vehicle, 3) fluoxetine, 4) vehicle. Adolescente nakomelingen (bijna 35 dagen oud) werden getest in de 'open field test' en 'forced swim test' om angst- en depressie-gerelateerd gedrag te onderzoeken. Na de gedragstesten, werden de nakomelingen opgeofferd en hersenen werden geanalyseerd om het niveau van cel proliferatie (Ki67-labeling) in de dentate gyrus van de hippocampus na te gaan. Resultaten tonen aan dat postnatale fluoxetine blootstelling leidt tot een verhoging van depressie-gerelateerd gedrag in adolescente nakomelingen blootgesteld aan prenatale stress, maar het heeft geen effect in nakomelingen niet blootgesteld aan prenatale stress, onafhankelijk van geslacht en moedergedrag. Postnatale fluoxetine blootstelling leidt tot een gedeeltelijke verhoging van de cel proliferatie in de hippocampus van adolescente nakomelingen blootgesteld aan prenatale stress, maar leidt tot een verlaging van de cel proliferatie in de hippocampus van nakomelingen niet blootgesteld aan prenatale stress. Met dit onderzoek worden belangrijke effecten van postnatale fluoxetine behandeling op de ontwikkeling van nakomelingen aangetoond.



## 1. Introduction

Postpartum depression (PPD) is a growing health problem that affects 15% of women worldwide after delivering (WHO). PPD can have detrimental effects on the lives of women, children and their families [1]. Biological, psychological and social risk factors, such as a family history of psychopathology or past history of depression, stressful life events and difficulties in marital relationships, contribute to the development of PPD [2]. Because of the effects of PPD on the lives of women and their children, it is crucial to treat this disorder without harming the child. Currently, selective serotonin reuptake inhibitors (SSRIs) are commonly used for the treatment of PPD, because of their efficacy and minimal side effects on the mother [3]. However, the effect of postnatal exposure to SSRIs on behavior and related neurobiology during childhood and adolescence has yet to be fully determined. Evidence suggests that there is a link between maternal stress, anxiety, and depression during the perinatal period and behavioral and emotional problems in children [4]. In addition, maternal depressed mood has a significant impact on adolescent behavior. Therefore, this study uses an animal model of postpartum depression to investigate how prenatal maternal stress affects the development of **anxiety and depressive-like behavior** in **adolescent** offspring and how **postnatal maternal fluoxetine** exposure may further impact the developing offspring.

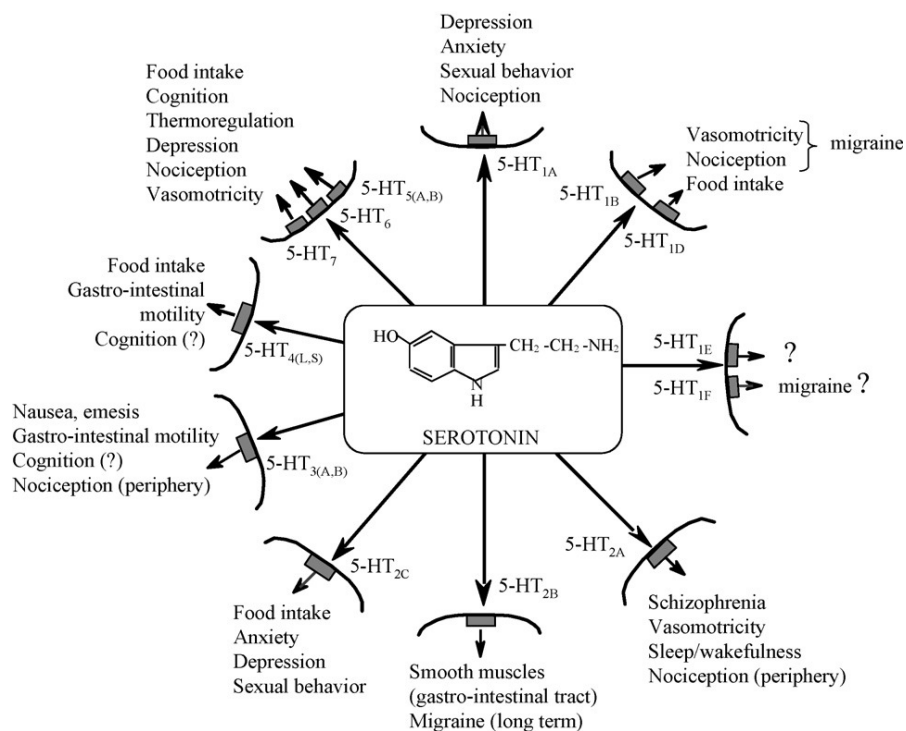
### 1.1 Pathophysiology of PPD

Depression is one of the most common mental disorders and is characterized by depressed mood, anhedonia (inability to experience pleasure from normally pleasure life events), abnormalities in appetite and sleep, low self-esteem, irritability and difficulties in concentrating [5]. Postpartum depression is a form of clinical depression that most commonly affects women after childbirth. Several limbic brain regions which regulate emotion, i.e. prefrontal cortex, hippocampus, amygdala and cingulated cortex, are involved in the pathophysiology of depression [5]. Commonly, depression occurs idiopathically and as a result of risk factors, such as stressful life events, cancers, endocrine abnormalities and side effects of drugs [5]. In addition, there is strong evidence that genetic predispositions interact with environmental risk factors to initiate depressive episodes [5].

The monoamines play an important role in depression and it has been hypothesized that the underlying mechanism of depression is a depletion of the levels of serotonin, norepinephrine (NE), and/or dopamine in the brain. This hypothesis seems to be supported by the mechanisms of antidepressant medications, which elevate the levels of these neurotransmitters in the central nervous system (CNS). However, more research

is needed to determine the primary mechanisms of specific monoamine systems implicated in depression [6].

The serotonergic (5-HT) system plays a key role in the modulation of mood, emotion, sleep and appetite and is involved in the control of behavioral and physiological functions. A decrease in the serotonergic neurotransmission has been suggested to play a primary role in the etiology of depression. The concentration of extracellular serotonin is controlled by its reuptake into the presynaptic cell [7]. Serotonin is synthesized from the essential amino acid L-tryptophan in serotonergic neurons located within the raphe nuclei. From these nuclei, 5-HT neurons project to nearly all parts of the CNS [8]. This extensive distribution of 5-HT fibers throughout the CNS is responsible for the numerous functions which can be controlled by 5-HT, such as food intake, sleep, thermoregulation, memory and learning, sexual behavior, locomotion, cardiovascular function, endocrine regulation and psychoaffective tone [9]. Several 5-HT receptor types have been characterized (Figure 1). Many of these receptors may be implicated in stress-related disorders, but only the 5-HT<sub>1A</sub> receptor plays an essential role in depressive disorders [10].



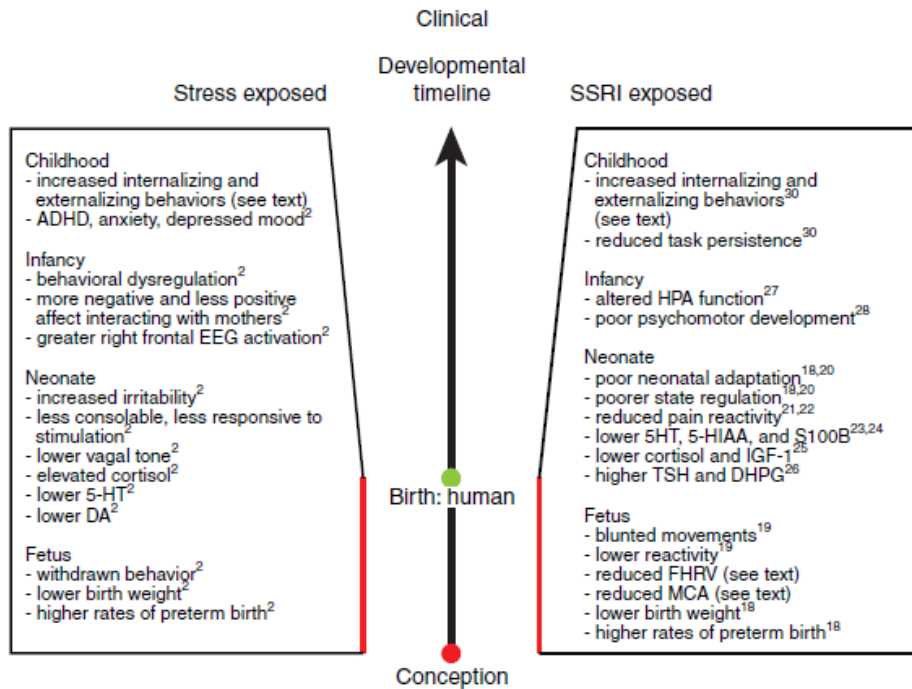
**Figure 1: Schematic overview of the 5-HT receptor types and their involvement in physiological, phychoaffective and phycho pathological conditions [11].**

## 1.2 Effects of PPD on offspring development

PPD affects 15% of women in the first year after delivering (WHO). Maternal stress and depression may have serious consequences for the developing child. Stress can be caused by changes in the environment, internal or external, that disrupt the

maintenance of homeostasis. When an organism is exposed to stress, several processes in the central nervous system (CNS) are initiated, primarily through the hypothalamic-pituitary-adrenal (HPA) axis. Stress stimulates the paraventricular nucleus (PVN) of the hypothalamus and corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) are secreted into the median eminence. CRH and AVP reach the anterior pituitary gland, via the portal vessels, where they induce the release of adrenocorticotrophin hormone (ACTH) into the circulation. ACTH is transported to the adrenal gland cortex, where it stimulates glucocorticoid-producing cortex cells. These cells secrete glucocorticoids, such as cortisol (in humans) or corticosterone (CORT; in rodents) into the circulation [12]. Chronic stress commonly results in glucocorticoid and CRH hypersecretion. In susceptible individuals, chronic stress can lead to long lasting HPA responses which can result in mood disorders such as depression and anxiety [13].

In humans, maternal stress, which can lead to maternal depression and anxiety, can have long-term effects on the offspring. Antenatal maternal stress can lead to neurobehavioral disturbances, reduced birth weight, and increased chance of premature births [14]. In addition, maternal depression can have several consequences in the newborn: irritability, reduced vagal tone, lower dopamine and serotonin levels, and increased cortisol and norepinephrine levels [14]. Maternal stress also affects cognitive and behavioral outcomes and leads to delayed motor development, delayed mental development and poorer language abilities in children [15] (Figure 2). A recently published study found that maternal depression during pregnancy was associated with a fourfold increase in the risk for violent behaviors in children [16]. However, the mechanisms by which maternal stress affects fetal and child development has to be investigated.



**Figure 2: Clinical findings associated with exposure to maternal stress or to SSRIs. Red vertical lines represent the time of exposure. ADHD, attention-deficit disorder; DA, dopamine; 5-HT, serotonin, DHPG, dihydroxyphenylglycol; EEG, electroencephalography; FHRV, fetal heart rate variability; IGF-1, insulin-like growth factor-1; MCA, middle cerebral artery; S100B, S100 calcium-binding protein B; HPA, hypothalamic-pituitary-adrenal; TSH, thyroid-stimulating hormone [17].**

In rats, persistent gestational stress in the third-trimester reduces glucocorticoid receptor levels in the hippocampus and hypothalamus, increases the basal activity of the HPA axis, and reduces synaptic density [14, 18]. In addition, maternal stress results in developmental differences in amygdalar nuclei, which may predispose these rats to fear-related behaviors [19]. In nonhuman primates, maternal stress results in reduced birth weights [14]. In these animals, stress during the pregnancy also resulted in behavior disturbances at juvenile, adolescent and adult ages, including impaired attention, increased anxiety and HPA-axis sensitivity [14]. In addition, exposure to maternal stress during early and late pregnancy in rhesus monkeys was correlated with a decrease of hippocampal volume and an inhibition of neurogenesis in the dentate gyrus of the hippocampus [20]. Several animal studies have indicated that adult offspring of prenatally stressed mothers showed increases in anxiety and depression-related behavior, e.g. decreased locomotor activity in the open field test, increased immobility in the forced swim test and decreased exploration in the elevated plus-maze test [21] (Figure 3). However little work has investigated effects of maternal depression on adolescent behavior.

### ***1.3 Treatment of PPD***

A number of pharmacological treatments are available for mood disorders. The most common antidepressants are (1) tricyclic compounds, which inhibit the reuptake of both 5-HT and NE by transporters; (2) selective serotonin reuptake inhibitors (SSRIs), which block the reuptake of 5-HT; (3) NE-selective reuptake-inhibitors, which block the reuptake of NE; and (4) monoamine-oxidase (MAO) inhibitors, which diminish the enzymatic degradation of 5-HT and NE. Other non-pharmacological treatments for mood disorders include psychotherapy, cognitive behavioral therapy, electroconvulsive therapy and exercise [22].

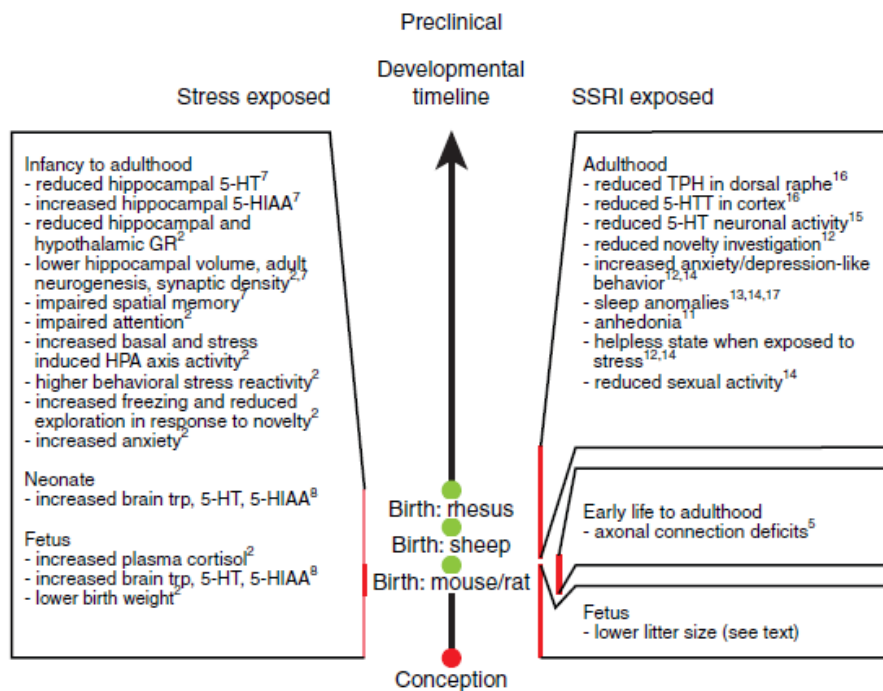
SSRIs such as Fluoxetine (Prozac) are commonly used in women and appear to be more effective in treating symptoms of depression in women than in men. This can be due to the fact that estrogens influence brain systems involved in the activity of serotonin [23]. During pregnancy and the postpartum period a growing number of women are taking SSRI medications [24]. Many breastfeeding women with depressive symptoms may not require pharmacotherapy, but in patients with severe PPD, medications may be helpful. A large number of studies have shown that these drugs are relatively safe in breastfeeding women [25], however the drug and its metabolites can be present in breast milk [26]. Therefore, it is important to weigh the risks of treatment with antidepressant medications on mother and offspring.

### ***1.4 Consequences of antidepressant treatment on development***

It is important to discriminate between antidepressant treatment and the maternal condition that requires treatment to understand the impact of these medications on fetal development. In humans, perinatal antidepressant treatment, regardless of maternal mood state, is associated with lower birth weight, more preterm births and neurobehavioral disturbances in the infants [27]. In addition, several neonatal neurobehavioral disturbances can occur as a result of gestational exposure to SSRIs, like tachypnea, temperature instability, irritability, weak or absent cry, poor feeding and reduced heart rate variability [27]. All these findings suggest that early neurobehavioral deficits and SSRI exposure may lead to an increased susceptibility to behavioral irregularities in early childhood (Figure 2). However, more research is needed to determine the effects of postnatal exposure to SSRIs on child development.

Several animal studies have focused on determining the effects of perinatal antidepressant exposure in offspring. A recent study examined how SSRIs affect the development of the fetus. It revealed that fetal exposure to fluoxetine was related to an increase in mortality rates, in a dose dependent manner. Most of the mice died postnatally due to severe heart failure as a result of dilated cardiomyopathy. Also, long-

term alterations in serotonin transporter levels were found in the raphe nucleus of these mice. Interestingly, fetal exposure to fluoxetine alone resulted in anxiety-like behavior in adult offspring [28]. Other research has demonstrated that offspring of pregnant rats exposed to high doses of fluoxetine exhibit decreased levels of 5-HT receptors in the hypothalamus, altered density of 5-HT transporters, decreased 5-HT levels in the central nervous system and down-regulation of cortical [<sup>3</sup>H]imipramine receptors [29]. How these findings correlate with the development of the human brain and behavior remains unknown (Figure 3).



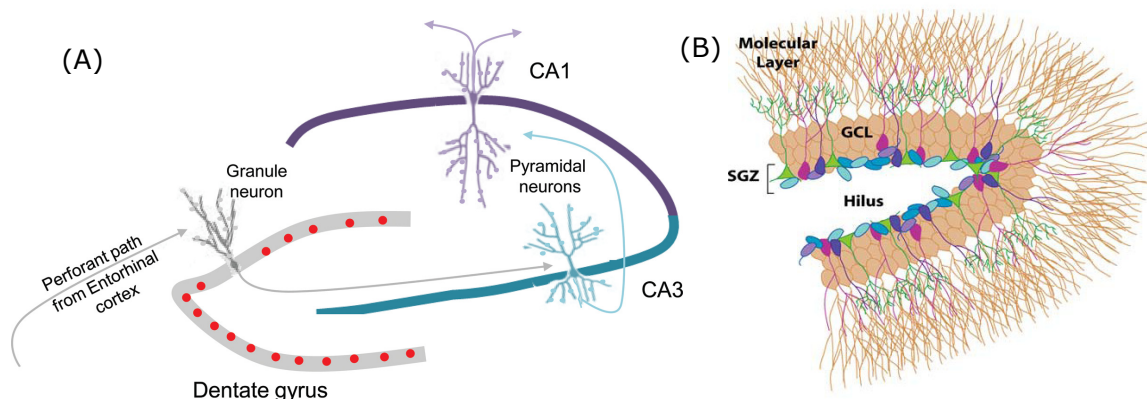
**Figure 3: Preclinical findings associated with exposure to maternal stress or to SSRIs. Red vertical lines represent the time of exposure, the light red stands for the time of exposure in rhesus macaques and the dark red stands for the time of exposure in the rat. 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; 5-HTT, serotonin transporter; GR, glucocorticoid receptor; HPA, hypothalamic-pituitary-adrenal; TPH, tryptophan hydroxylase; trp, tryptophan [17].**

### ***1.5 Neurobiological correlates of stress, depression and antidepressant medications***

One primary neural region associated with stress, depression and antidepressant medication is the hippocampus (Figure 4) [30]. The hippocampus is a regulator of the HPA axis and as such plays a primary role in the response to stress and stress-related diseases [31]. Recent work has demonstrated that depression and antidepressant medications may act via changes in neurogenesis in the adult hippocampus, particularly via alterations in cell proliferation in the dentate gyrus (DG) of the hippocampus [30].

Reductions in cell proliferation are shown in animal models of stress and depression [32]. This suggests that suppression of cell proliferation in the hippocampus contributes to the pathogenesis of depression. Loss of hippocampal volume is well documented in depressed patients [33]. This volume loss correlates with disturbed hippocampal function, such as cognitive impairments [33]. In addition, antidepressant treatment stimulates hippocampal cell proliferation and reverses the inhibitory effect of stress [34]. Although, there are sex differences in the responsiveness to antidepressants in relation to cell proliferation, research has shown that fluoxetine increases cell proliferation in adult male rats but not in peri-pubescent males or female rats at any age or stage in the estrous cycle. Only higher doses of fluoxetine can induce increases in hippocampal cell number in the female rodent [35].

However very little is known about the role of maternal depression and postnatal antidepressant on neurogenesis in adolescent offspring. Several studies have shown that maternal stress alone suppresses cell proliferation in the DG of adult offspring [36]. Some work has demonstrated that maternal stress already induces a decrease in cell proliferation at the embryonic stage of the developing brain [37]. Interestingly, there is an age-dependent effect of maternal stress on cell proliferation in females, as decreases are only observed when females reach senescence [38].



**Figure 4: (A) Schematic diagram of the hippocampus showing a mature granule neuron of the dentate gyrus and mature pyramidal neurons of CA3 and CA1 areas, and also their main axonal connections [39]. (B) Structures of the dentate gyrus, with the subgranular zone (SGZ) laying at the border of the granule cell layer (GCL) and hilus. The SGZ contains cells at various stages of neurogenesis [30].**

## **1.6 The effects of serotonin on brain development and neurogenesis**

Serotonin plays not only an important role in depression and adult brain plasticity, but also during early stages of brain development [40]. Prenatal depletion of serotonin causes a delay in the onset of neurogenesis in serotonergic target regions, such as the DG of the hippocampus or the subventricular zone (SVZ), of adult rats [41]. Also,

depletion of serotonin levels during development may cause a loss of synapses. Early developmental disturbances of the serotonin system have the potential to alter brain structure [42]. Serotonin is an important factor in influencing the rate of neurogenesis in the DG [41]. The DG is provided with a very dense plexus of serotonergic fibers [43]. Depletion of serotonin in the brain of adult rats causes a decrease in the amount of cell proliferation in the SVZ and the DG [41]. As expected, increased levels of serotonin results in an increased proliferation rate in the DG [34]. These effects of serotonin on the regulation of adult neurogenesis in the DG are likely due to the actions of a number of 5-HT receptors. *In vitro* experiments have shown that when fibroblasts are transfected with the 5-HT<sub>1A</sub> receptor and cultured in a medium containing the 5-HT<sub>1A</sub> antagonist (8-OH-DPAT), the cell proliferation rate is increased [44]. In addition, activation of 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptors also increases adult neurogenesis in the DG, while agonists of the 5-HT<sub>1B</sub> receptors increase neurogenesis only after depletion of serotonin [45]. 5-HT<sub>4</sub>, 5-HT<sub>6</sub> and 5-HT<sub>7</sub> receptors may also be involved in mediating the effects of serotonin on cell proliferation in the DG. These receptors trigger the cAMP cascade, which consequently increase the expression of brain-derived neurotrophic factor (BDNF). BDNF has no direct effect on the rate of cell proliferation in the DG, but may increase the release of serotonin, which in turn may stimulate cell proliferation by increased activation of 5-HT<sub>1A</sub> receptors [46].

### ***1.7 Aim and hypothesis***

To our knowledge, no research has studied the effect of fluoxetine treatment on development of anxiety and depression-related behavior or hippocampal cell proliferation in adolescent offspring in a model of maternal depression. **We hypothesized that (1) maternal stress results in anxiety- and depressive-like behavior and alters neurobiology in offspring, and that (2) postnatal fluoxetine exposure protects against these changes in offspring.** The aim of the present study was to determine how maternal stress and fluoxetine affects (1) **anxiety- and depressive-related behavior** and (2) **hippocampal cell proliferation** in **adolescent** offspring. Knowledge of the effects of maternal depression and antidepressant treatment during the postnatal period is needed to ameliorate treatment and intervention options, so that neurodevelopmental outcomes can be improved.



## 2. Materials and methods

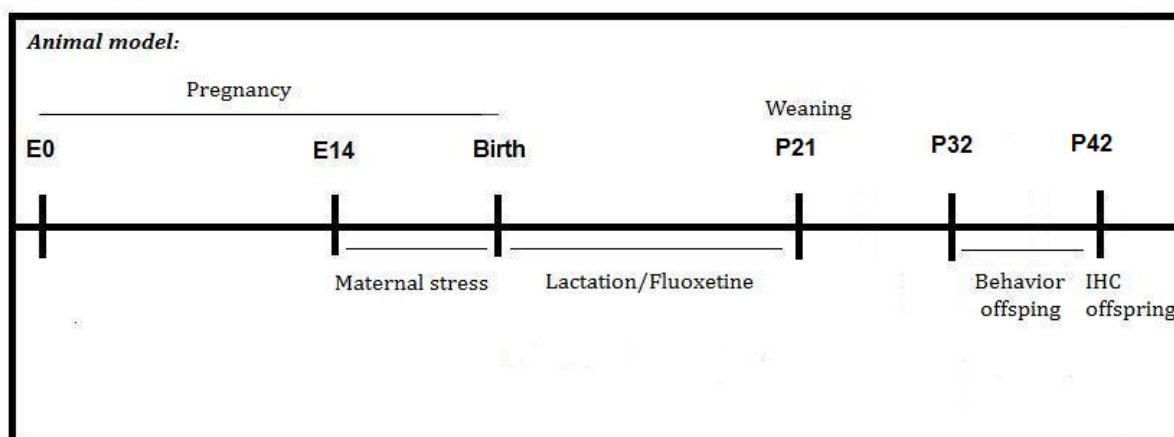
### 2.1 *Experimental set-up and animal model*

#### 2.1.1 Animals

Twenty-eight adult female Sprague-Dawley rats (250-300g; Charles River Laboratories) were used in the present study. Females were housed in opaque polyurethane bins (48x27x20 cm) with corn cob bedding. Rats were kept under standard laboratory conditions in a 12h light/dark schedule (lights on at 07:00 h) with access to rat chow (Sniff) and tap water *ad libitum*. All experiments were approved by the Animal Ethics Board of Maastricht University in accordance with Dutch governmental regulations (DEC 2008-157 and DEC 2008-158). All efforts were made to minimize the pain and stress levels experienced by the animals.

For breeding, one female and one male were paired in a wire mesh cage. Upon release of a vaginal plug, females were individually housed in polyurethane bins. On gestation day (GD) 15 dams were randomly assigned to stress (n=12) or non-stress groups (n=16). Dams in the stress group were restrained in clear tubes under bright light during the last week of pregnancy three times daily for 45 minutes between GD15-20 and twice on GD21.

One day after birth (birth day = day 0), dams and litters were weighed and all litters were culled to 5 males and 5 females. Stress and non-stressed dams (with offspring) were randomly assigned to one of two treatment groups: fluoxetine (5mg/kg/day) or vehicle. Therefore in total there were four groups of dams: 1) Maternal Stress + Vehicle (MSV), 2) Maternal Stress + Fluoxetine (MSF), 3) Control Fluoxetine (CF), and 4) Control Vehicle (CV). Treatment was given to the mothers throughout lactation, via osmotic minipump (Alzet Osmotic pumps, Cupertino, CA, USA) implants, beginning the day after birth and continuing until weaning. At weaning offspring were weighed and a maximum of 2 males and 2 females were used from each litter for behavioral testing and immunohistochemistry. Offspring were tested on behavioral tasks between 32-39 days of age and were sacrificed for brain harvest at least two days after behavioral testing (Time line: Figure 5).



**Figure 5: Experimental procedures for the animal model. At E14 maternal stress is induced until E21 (birth). Fluoxetine treatment is given to the mother after birth until weaning (P21). From P32 until P39, the offspring is subjected to behavioral tasks. At P42, the offspring is sacrificed and brains are used for immunohistochemistry (IHC).**

Eight different groups of offspring were used for behavioral testing (Table 1) and immunohistochemistry (IHC). Siblings from each litter were housed together for a total of 4 offspring per cage. All animals were tested on behavioral tasks. For assessment of hippocampal cell proliferation, 5 animals per group were used (1 male and 1 female from each litter).

**Table 1: Different experimental groups**

Experimental group	Sex	Number
Maternal stress + Fluoxetine [MSF]	Male	11
	Female	10
Maternal stress + Vehicle [MSV]	Male	9
	Female	9
Control Fluoxetine [CF]	Male	10
	Female	9
Control Vehicle [CV]	Male	10
	Female	9

## 2.2 Treatment

Fluoxetine treatment was administered via osmotic minipumps (Alzet Osmotic pumps, Cupertino, CA, USA). Implants were filled with either fluoxetine, dissolved in 50% propylenediol in saline (5 mg/kg/day), or with vehicle as previously described [47]. Minipumps were implanted subcutaneously in the dorsal region of the mothers under mild isofluorene anesthesia on post-partum day 1 (P1).

## **2.3 Maternal behavior**

Maternal behavior was assessed on P2 to P7 as previously described [48]. The duration of the following maternal behaviors were observed during each testing period: licking/grooming (body licking and genital licking with the dam off the pups); licking/grooming/nursing (body licking and genital licking with the dam on the pups); arched-back nursing (the dam arches her back while pups nurse); "blanket" nursing (the dam lies on the pups); passive nursing (the dam lies either on the back or the side while pups nurse); nest building; and time off pups. Scoring of maternal behaviors occurred twice a day for 5 min. Animals were scored once in the morning (between 8:30 a.m. and 10:30 a.m.) and once in the afternoon (between 13:30 p.m. and 15:30 p.m.) with at least 3 h between the sessions. Data were then aggregated across days and calculated as percent time spent in each behavior.

## **2.4 Behavior testing**

To assess how maternal stress and fluoxetine treatment affect the development of anxiety- and depressive-like behavior in adolescent offspring, offspring were tested on the open field test and the Porsolt forced swim test as previously described [49]. Animals were tested in one test per day between 9 a.m. and 2 p.m. Litter mates were tested consecutively in random order.

### **2.4.1 The open field test (OFT)**

The open field test (OFT) was used to study anxiety-like behavior and locomotor activity as rodents show anxiety-like behavior in novel, open spaces [50]. The open field test consisted of a 100 cm x 100 cm area divided into central and peripheral areas with 40 cm high walls. The central portion of the OFT was 50 cm x 50 cm. The apparatus was placed in a dimly lit room. Offspring were tested on the OFT between the ages of 32 and 34 days. At the beginning of the test a rat was placed in the center of the field and behavior was recorded for five minutes. All animals were tested once between 9:30 a.m. and 2 p.m. A video-tracking system (Anymaze; Stoelting) was used to score the distance travelled, mean velocity, number of entries into the central and peripheral areas, and total amount of time in the central and peripheral regions. An observer blind to the groups scored the number of rearing postures and the number of fecal boli from each animal. The apparatus was cleaned with 70% ethanol and dried between rats. Decreased anxiety-like behavior was measured as an increase in the amount of time in the central zone of the open field. Increased distance travelled was considered an index of locomotor activity.

## **2.4.2 The Porsolt forced swim test (FST)**

The Porsolt forced swim test (FST) was used to assess depressive-like behavior in the adolescent offspring as previously described [49]. This test measures the behaviors of the animal in response to an inescapable environment. The apparatus consisted of a vertical cylindrical glass tank (height 50 cm x diameter 20 cm) filled to a depth of 20cm with tap water at  $27 \pm 1^\circ\text{C}$ . This depth was sufficient to ensure that the offspring could not touch the bottom of the glass tank with their hind paws and has previously been used with adolescent offspring [51]. The animals were placed in a cylindrical glass tank filled with warm water for ten minutes, towel dried and returned to their home cage. Sessions were videotaped and scored using the Best Collection System (Educational Consulting Inc.) by an observer blind to the different groups. Offspring were tested on the FST between the ages of 37 and 39 days. Behaviors scored in the FST were (1) immobility – floating with the absence of any movement and (2) struggling – quick movements of the forelimbs such that the front paws break the surface of the water. The number of escape attempts was also recorded. Escape was defined as swimming to the bottom of the tank and pushing up. Increased duration of time spent immobile during testing is indicative of increased depressive-like behavior.

## **2.5 Immunohistochemistry (IHC)**

Two to three days after the last behavioral tests, between 10 a.m. and noon, offspring were deeply anesthetized, weighed, blood was drawn via cardiac puncture, decapitated and brains were removed. Half of the brain was used for IHC, the hippocampus of the other half was used for further analysis not included in the present study. Brains were post-fixed in 4% paraformaldehyde for 48 hours, then cryoprotected in 30% sucrose/phosphate-buffered saline solution for up to one week, frozen on dry ice and kept at  $-80^\circ\text{C}$ . Brain tissue was sliced in  $40\mu\text{m}$  sections on a cryostat (Leica). Slices were saved in tissue antifreeze solution and maintained at  $-20^\circ\text{C}$ . Every 6<sup>th</sup> section throughout the hippocampus was used for immunohistochemistry.

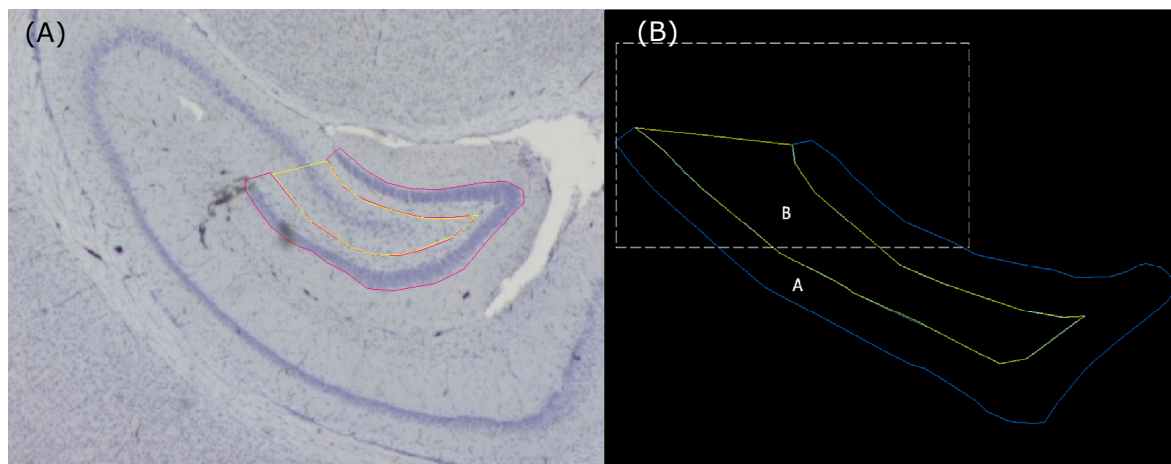
### **2.5.1 Ki67 staining**

Levels of new cell proliferation were assessed in the dentate gyrus of the hippocampus using an endogenous marker, Ki67. Tissue was stained for expression of Ki67 as previously described [52]. Free-floating sections were rinsed between steps with PBS (phosphate buffered saline). Sections were incubated in 0.3%  $\text{H}_2\text{O}_2$  for 30 minutes at room temperature to block endogenous peroxidase activity. Tissue was then incubated overnight in rabbit anti-Ki67 immunoglobulin G primary antibody (1:500; Vector Laboratories) in 0.4% Triton-X in PBS at  $4^\circ\text{C}$ . The next day, sections were incubated overnight in donkey anti-rabbit biotinylated antibody (1:500; Jackson ImmunoResearch,

Suffolk, UK) at 4 °C. Brain sections were further processed for immunohistochemistry by using the avidine-biotine complex (ABC Elite kit; 1:1000; Vector laboratories) for 4 h. DAB (3,3-diaminobenzidine; Sigma, The Netherlands) was used as a substrate to obtain a color reaction. Sections were mounted on gelatin-coated slides, dried overnight, counterstained with Cresyl Violet acetate, dehydrated and coverslipped with Permount (Fisher Scientific, Hampton, NH, USA).

### 2.5.2 Design-based stereology

Design-based stereology was performed for volume measurements and cell counts. A stereological computer microscopy system and StereoInvestigator software (MicroBrightField, Williston, VT, USA) were used. The microscopy system consisted of a modified Olympus BX51 microscope (Olympus, Tokyo, Japan) with PlanApo and UPlanApo objectives 2x (Olympus; N.A. = 0,06), 10x (Olympus; N.A. = 0,25), 40x (Olympus; water; N.A. = 1,15), three-axis high accuracy computer controlled motor specimen stage (4x4 Grid Encoded Stage Ludle Electronic Products, Hawthorne, NY, USA), linear z-axis position encoder (Ludl), and a 3CCD color video camera (Hitachi, Japan). The regions of interest, the dentate gyrus and the hilus of the hippocampus, were delineated with a 4x magnification on live microscopic images (Figure 6).



**Figure 6: (A) Delineation of the hippocampus on a live image. (B) The hippocampus is divided into the granular cell layer + subgranular zone (A) and the hilus (B)**

Volumes were estimated, according to Cavalieri's principle, by multiplying the sum of the surface areas with the section thickness (40 $\mu$ m), with a factor of 2 because half of the brains were used and a factor of 6 since every sixth section was used. Ki67-positive cells were counted with the optical fractionator method [53]. Counting frames (140 x 140  $\mu$ m) were created by the software and placed at the intersections of a grid (140 x 140  $\mu$ m) that had been placed over the sections. The counting frames were replaced systematically by stepwise movements in x- and y-directions. The number of positive cells was calculated by multiplying the sum of the cell counts with a factor of 6 since

every sixth section was used and also with a factor of 2 for an indicator of cell proliferation in the entire hippocampus. Cell densities were calculated by dividing the number of positive cells by volume.

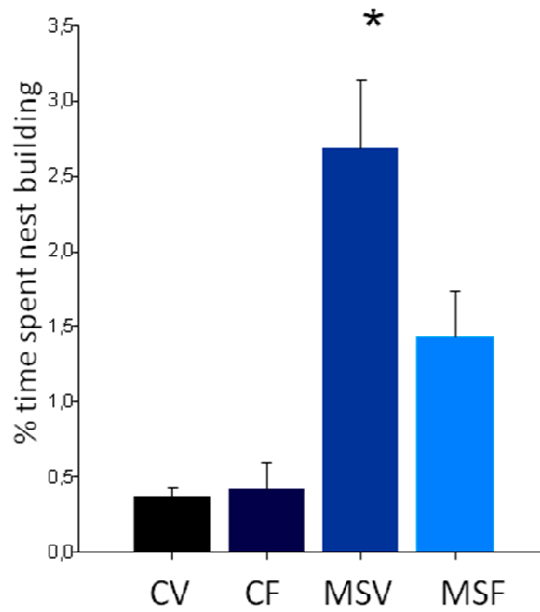
### **2.5.3 Statistical analyses**

Analysis of maternal pup-directed behaviors, FST measures, OFT measures, volume estimates of the GCL/SGZ and hilus, and Ki67-ir (Ki67-immunoreactive) cell densities of the GCL/SGZ and hilus was done by using a repeated measures analysis of variance (ANOVA) test with treatment and sex as independent factors. Analysis of body weight change was done by using a one-way ANOVA test with treatment and sex as independent factors. Correlations were conducted between maternal behavior and the FST, OFT, volume estimates of the GCL/SGZ and hilus and Ki67-ir cell densities. An analysis of covariance was done on scores of the OFT and FST with age, weight (FST) and order of testing as covariates. Post hoc comparisons utilized the Fisher LSD test. The accepted level of statistical significance was  $p < 0.05$  for all analyses. All calculations were done using the Statistica 9 Software.

## **3. Results**

### **3.1 Maternal behavior**

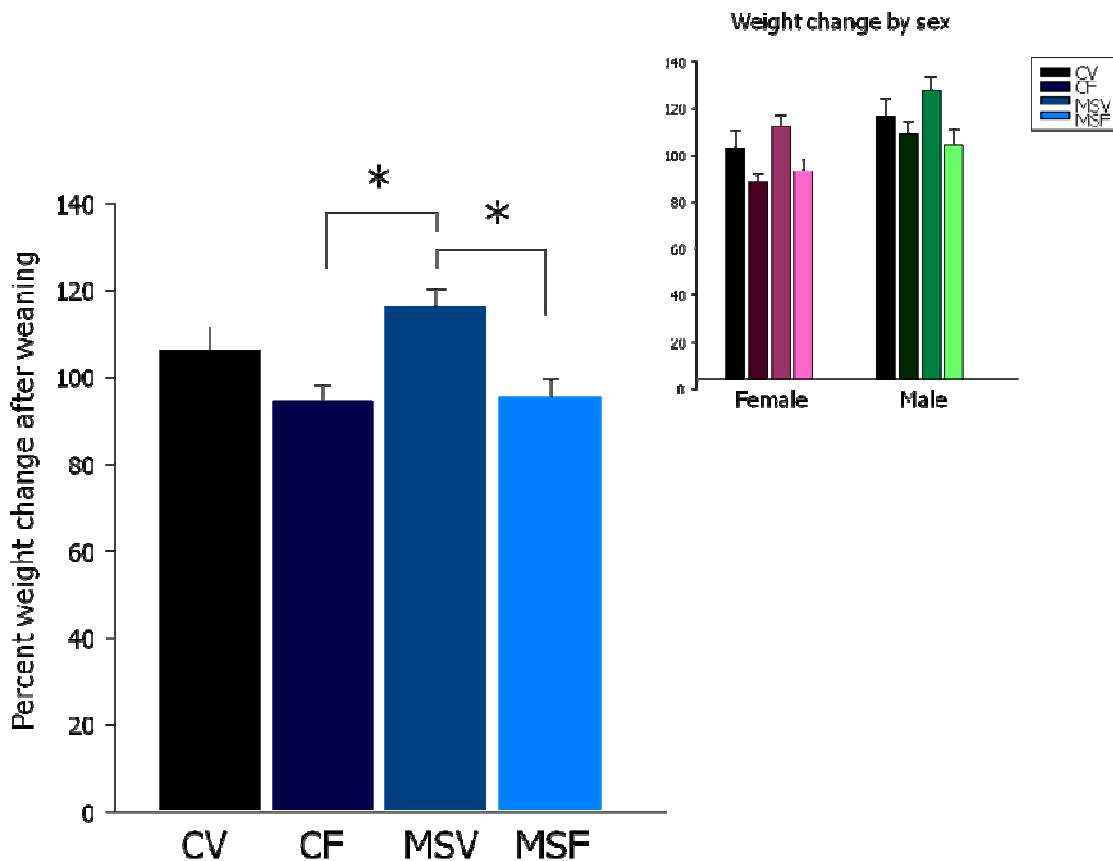
A repeated measures ANOVA on maternal pup-directed behaviors demonstrated that there are significant differences in the time the dams spent in certain behaviors ( $F_{(15, 190, 88)} = 3.0904$ ,  $p = 0.0016$ ). Post-hoc tests revealed that dams spent significantly more time in arched-back nursing and blanket nursing than with licking/grooming/nursing and passive nursing ( $0.03 \leq p \leq 0.00003$ ). One-way ANOVA demonstrated that there was a significant main effect of treatment on time spent nest building ( $F_{(3,73)} = 14.283$ ,  $p = 0.001$ ; data not shown). Post-hoc tests revealed that dams in the MSV group show a significant increase in the amount of nest building compared to all other groups ( $0.001 \leq p \leq 0.002$ ; Figure 7). Dams in the MSF group also spent significantly more time nest building than animals in the CV and CF groups ( $0.008 \leq p \leq 0.01$ ). There were no other significant differences between groups in the other pup-directed behaviors such as licking/grooming, or arched-back nursing ( $0.08 \leq p \leq 0.5$ ).



**Figure 7: Maternal stress significantly increases the time dams spent nest building, compared to all other groups ( $0.001 \leq p \leq 0.002$ ).**

### ***3.2 Body weights***

The weight of offspring was measured prior to behavioral testing (P28) and the day of sacrifice (P42). A one-way ANOVA on the percentage of weight gain between P28 and P42 resulted in a significant main effect of treatment ( $F_{(3,68)}=5.2722$ ,  $p=0.0025$ ) and sex ( $F_{(1,68)}=14.565$ ,  $p=0.00029$ ) even when controlling for age. Post hoc comparisons on main of treatment demonstrated that offspring in the MSV group have a significantly higher percentage of weight gain compared with animals in the CF and MSF group ( $0.002 \leq p \leq 0.003$ ; Figure 8). In turn, males gained significantly more weight than females ( $p \leq 0.001$ ; Figure 8). There were no other significant differences between groups and no significant interaction effect of sex and treatment ( $F_{(3,68)}=0.13875$ ,  $p=0.9365$ ).

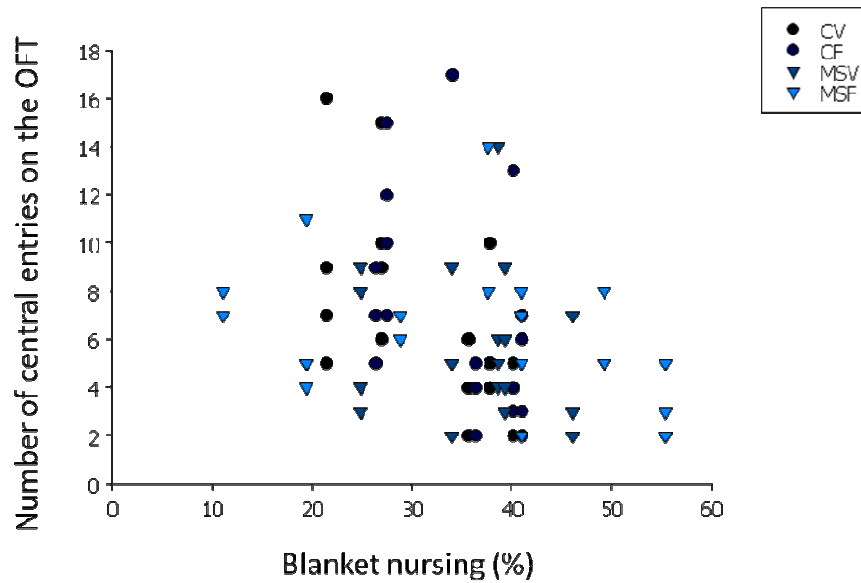


**Figure 8: Mean  $\pm$  SEM of the percentage of weight gain between P28 and P42. Offspring in the MSV group gained significantly more weight than animals in the CF and MSF group ( $0.002 \leq p \leq 0.003$ ). In turn males gained more weight than females ( $P \leq 0.001$ ).**

### ***3.2 The open field test***

The open field test was used to determine anxiety-like behavior and motor ability. There were no significant differences between groups in the time spent in periphery of the open field, time spent in center of the open field, total distance travelled, mean speed, peripheral and central entries and the amount of rearing ( $0.5 \leq p \leq 0.8$ ). There was a significant negative correlation between the number of entries into the center of the open field and percent of time blanket nursing ( $r=0.38$ ;  $p=0.001$ ; Figure 9). In addition, there was a tendency toward a negative correlation ( $r=-.1792$ ;  $p=0.078$ ) between time spent in the center of the open field and the time spent nursing, indicating that more time the offspring spent in the center of the open field was related to less time of nursing. There were no other significant differences between groups or correlations ( $0.4 \leq p \leq 0.9$ ).

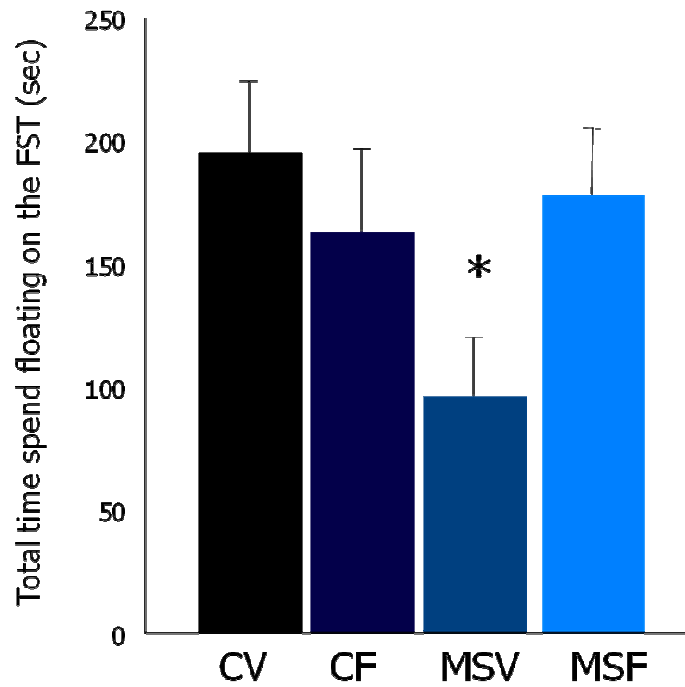




**Figure 9: Correlation between the percentage of time a dam spent blanket nursing and the number of central entries on the OFT. The percentage of time spent in blanket nursing was negatively associated with the number of central entries on the OFT. ( $r=-0.38$ ,  $p=0.001$ ).**

### ***3.3 The Porsolt forced swim test***

The Porsolt forced swim test was used to determine depressive-like behavior. Repeated measures ANOVA on time spent struggling and immobile, controlling for weight, resulted in a significant main effect of treatment ( $F_{(3,68)}=2.7605$ ,  $p=0.04875$ ) and behavior ( $F_{(1,68)}=6.5965$ ,  $p=0.01242$ ). There was also a significant interaction effect between behavior and treatment ( $F_{(3,68)}=3.3808$ ,  $p=0.02310$ ). Post hoc test revealed that animals in the MSV group were floating significantly less compared to all other groups ( $0.01 \leq p \leq 0.03$ ; Figure 10). There were no other significant interactions or differences in floating or struggling between the other groups ( $0.1 \leq p \leq 0.9$ ). There were no significant differences in number of escape attempts between the groups ( $0.1 \leq p \leq 0.3$ ). Also, there were no significant correlations between maternal behaviors and measures of the FST ( $0.1 \leq p \leq 0.9$ ).



**Figure 10: Mean  $\pm$  SEM of the time of floating in the FST. Animals in the MSV group spent significantly less time floating compared with animals in the CV group, regardless of sex or weight ( $0.02 \leq p \leq 0.05$ ). \* Significantly different from all other groups.**

### ***3.4 Hippocampal cell proliferation***

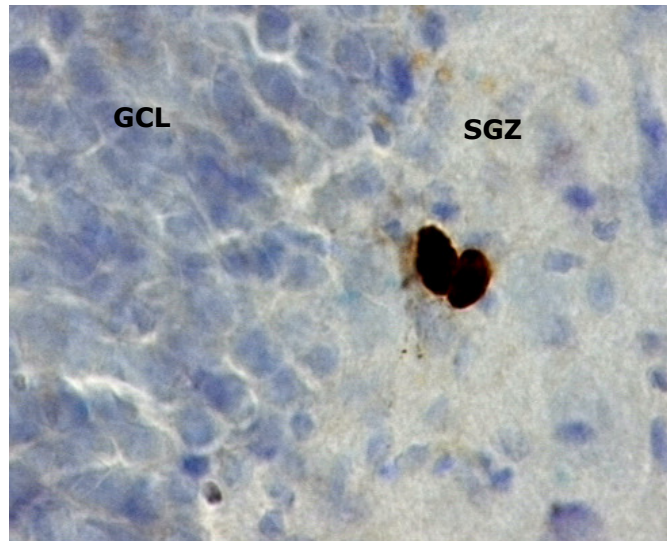
Volumes of the GCL/SVZ of the dentate gyrus and hilus were measured to determine if there are differences with maternal stress and/ fluoxetine treatment. A repeated measures ANOVA on the volume of the GCL/SGZ and hilus revealed a significant main effect of treatment ( $F_{(3,26)}=3.5166$ ,  $p=0.02905$ ) and region (GCL versus hilus) ( $F_{(1,26)}=664.55$ ,  $p=0.000$ ). There was also a interaction effect of treatment and region ( $F_{(3,26)}=3.3959$ ,  $p=0.03274$ ). Post hoc test revealed that adolescent offspring in the MSV group had a significantly smaller volume of the GCL/SGZ than adolescent offspring in the other groups ( $0.008 \leq p \leq 0.05$ ). Offspring in the MSV group also had a significantly smaller hilus compared with offspring in the MSF and CF group ( $0.000003 \leq p \leq 0.03$ ; Table 2). Therefore cell densities were used to assess the degree of hippocampal cell proliferation.

A repeated measures ANOVA on the density of Ki67-ir cells (Figure 11) resulted in a significant main effect of treatment ( $F_{(3, 26)}=8,8796$ ,  $p=,00032$ ; Figure 12). Post hoc tests revealed that adolescent offspring in the CV group had significantly higher densities of Ki-67-ir cells in compared to all other groups ( $0.001 \leq 0.01$ ) regardless of region (GCL/SVZ or hilus). There was also a tendency for MSF to be higher than the CF group ( $p=0.056$ ). There was also a significant main effect of region ( $F_{(1, 26)}=325.02$ ,  $p=,00000$ ) with greater Ki67-ir cell density in the GCL/SVZ region of the hippocampus than in the hilus. There was no significant interaction between treatment and region in Ki67-ir cell

density in the hippocampus ( $p=0.321$ ). In addition, there was a significant negative correlation between the percentage of time a dam spent in arched-back nursing postures and Ki67-ir cell densities in the GCL/SGZ ( $r=-0.3396$ ,  $p=0.046$ ; Figure 13). There were no other significant correlations between maternal behaviors, measures on the FST or OFT, and Ki67-ir cell density in the GCL or hilus ( $0.1 \leq p \leq 0.9$ ).

**Table 2: Mean ( $\pm$ SEM) volume ( $\text{mm}^3$ ) estimates of the GCL/SGZ and the hilus of the hippocampus. There were significant differences in volume. Offspring in the MSV group had a significantly smaller volume of the GCL/SGZ and hilus than the other groups ( $0.008 \leq p \leq 0.05$ ).**

Condition	Mean ( $\pm$ SEM) GCL/SGZ	Mean ( $\pm$ SEM) Hilus
CV	3,74 $\pm$ 0,32	2,52 $\pm$ 0,28
MSV	2,99 $\pm$ 0,30	2,04 $\pm$ 0,28
MSF	4,13 $\pm$ 0,24	2,93 $\pm$ 0,25
CF	3,88 $\pm$ 0,15	2,93 $\pm$ 0,14



**Figure 11: Representative picture of Ki67-ir cells in the subgranular zone of the hippocampus.**

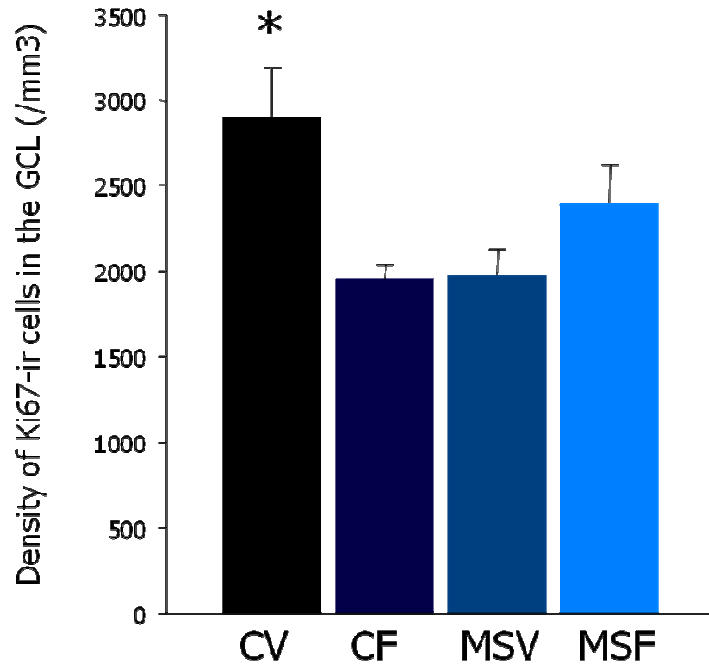
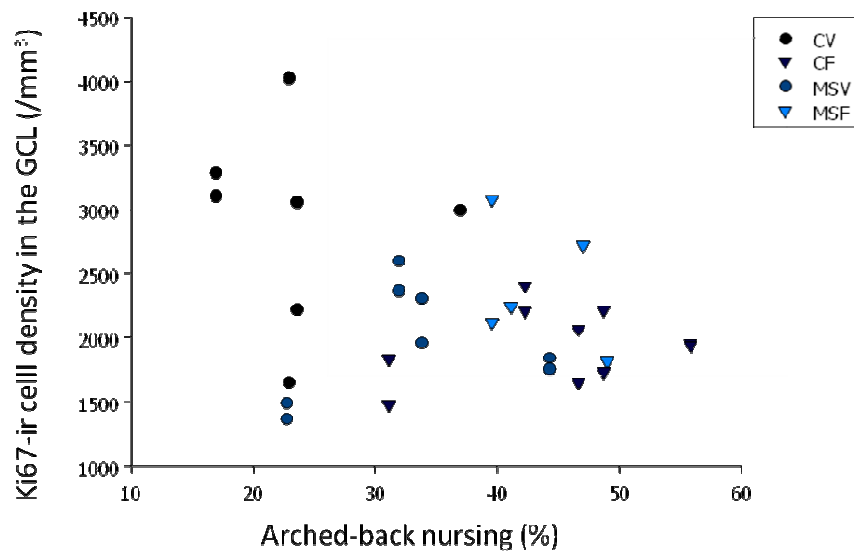


Figure 12: Mean  $\pm$  SEM of the Ki-67-ir cell densities in the GCL/SGZ. Animals in the CV group have a significant higher density of cell proliferation in the GCL/SGZ of the hippocampus than all other groups ( $0.001 \leq p \leq 0.01$ ). \* Significantly different from all other groups.



## **4. Discussion**

The results obtained in this study demonstrate that there are significant differences in the percentage of weight gain, depressive-like behavior, hippocampal size and hippocampal Ki67-ir cell densities in adolescent offspring in response to prenatal maternal stress and/or fluoxetine treatment. This study shows that animals exposed to maternal fluoxetine gained less weight than stressed offspring after weaning, regardless of maternal stress exposure. The Porsolt forced swim test revealed that there were significant differences in depressive-like behavior in adolescent offspring demonstrating that offspring exposed to maternal stress showed less depressive-like behavior than offspring in the control group. However, maternal fluoxetine treatment increased depressive-like behavior in adolescent offspring exposed to maternal stress, but had no effect on non-stressed offspring. Hippocampal cell proliferation was also significantly decreased in adolescent offspring exposed to stress and/or fluoxetine. A correlation was also found between time the dam spent in arched-back nursing and hippocampal cell proliferation (Ki67-ir cell densities) with more arched-back nursing resulting in lower Ki67-cell densities. Also, there was negative correlation between the time spent in blanket nursing and anxiety-like behavior. Furthermore, maternal stress seems to increase the time spent nest building. Administration of fluoxetine to stressed animals counteracts this increase partially.

### ***4.1 Maternal stress and fluoxetine treatment lead to changes in nest building behaviors***

This study shows that maternal stress increases the percentage of nest building and administration of postnatal fluoxetine treatment to stressed dams counteracts this increase partially. These data indicate that fluoxetine treatment has a direct effect on aspects of maternal care. Previous research has investigated the effects of reuptake inhibition of serotonin, as a consequence of fluoxetine exposure, on maternal behavior. This study showed that maternal behaviors, such as nest building, licking and touching are increased by a high dose of fluoxetine treatment in non stressed mothers [54]. Serotonergic signaling, which can be influenced by fluoxetine, in the CNS is important for good maturation and homeostatic modulation of neural circuits which are involved in emotions and physiological responses. Therefore, disturbances in the serotonergic gene expression can have a great impact on behavior [55]. By using dams with a specific disruption in serotonin neuron development, a recent study showed that serotonergic function is required for nurturing and survival of offspring [56]. Also, maternal stress affects maternal care directly. One study demonstrated that gestational restraint stress

alters maternal care in rats. Gestationally stressed dams show a reduction in the percentage of time spent arched-back nursing and nest building/grouping pups over P1 until P10 [57]. This is not in line with the findings in this study, where maternal stress seems to increase maternal care, by increasing the time of nest building, and fluoxetine treatment counteracts this increase partially. The differences between our results and others may be due to the fact that there were differences in the observation and quantification of maternal behaviors, as well as strain and species differences. More research is needed to understand the role of serotonin in maternal behaviors.

#### ***4.2 Fluoxetine treatment reduces weight gain***

The present study shows that stressed and non-stressed adolescent offspring exposed to maternal fluoxetine treatment, gain less weight than offspring which were exposed to the maternal stress. These data indicate that fluoxetine has a negative effect on weight gain in adolescent offspring. Previous research showed that a high dose of in utero fluoxetine results in a decrease of birth weight and also a reduction in weight gain during the preweaning period in rats [58]. A reduction in weight gain during the preweaning period can be a result of the involvement of 5-HT in the glucoregulation in the hypothalamus, because the CNS is responsible for controlling blood glucose in a very complex process involving several brain areas [59]. Some studies also investigated the effects of prenatal stress on body weight. One study has demonstrated that male and female pups from dams that experience prenatal stress and/or are on a high-fat diet weigh more beginning on postnatal day 7 compared with standard chow-control pups [71]. Pups exposed to maternal stress have impaired glucose tolerance, which increases the susceptibility to diet-induced obesity [60]. This suggests a possible mechanism by which prenatal stress can induce an increase in body weight, as shown in our study. Another study on the effects of prenatal stress on juvenile rat offspring outcomes showed that female offspring from gestationally stressed dams have a reduced rate of weight gain after weaning relative to their controls [61]. These findings were contrary to our data obtained in this study. Probably, there is an effect of maternal stress on the serotonergic system, which also is involved in glucoregulation. Possibly, the quantity, the duration and the time point of the maternal stress plays an important role in terms of weight gain. Also, strain and species differences can be important.

#### ***4.3 Postnatal fluoxetine increases depressive-like behavior in adolescent offspring exposed to maternal stress.***

This study demonstrates that there are significant differences in depressive-like behavior as a response to maternal stress and/or fluoxetine treatment. Adolescent

offspring exposed to maternal stress show less depressive-like behavior than offspring in the other groups. In addition, maternal fluoxetine treatment increases depressive-like behavior in adolescent offspring exposed to maternal stress, but had no effect in non-stressed offspring. Interestingly, maternal stress seems to protect against the development of depressive-like behavior in offspring, while fluoxetine treatment negatively influences depressive-like behavior in stressed offspring. Other research in mice has also showed that fluoxetine exposure during pregnancy and lactation results in increased immobility time, in female offspring, in the forced swim test at both P30 and P70 [62]. However, we found that fluoxetine alone didn't show the same effect as seen in stressed offspring. The negative effects of fluoxetine are probably due to the fact that fluoxetine treatment alters the HPA function and as such can lead to increases in cortisol concentration [63]. Although, the exact mechanisms by which fluoxetine influences depressive-like behavior in offspring are still not known. In rats, considerable evidence suggests that prenatal maternal stress programs the HPA axis as well as behavior, and that plasticity of the developing monoamine system in the brain underlies, in part, these changes. Because stress leads to a secretion of high levels of glucocorticoids, these 'stress hormones' probably play a significant role in the 'programming' of the developing brain [64]. Although in this study maternal stress seems to protect against the development of depressive-like behavior in adolescent offspring, this effect may be age dependent as other research did not find differences in immobility behavior in the forced swim test in adult offspring perinatally exposed to maternal stress [65].

#### ***4.4 Maternal stress leads to a reduction in hippocampal volume in adolescent offspring***

The present study shows there are significant differences in hippocampal volume in adolescent offspring as a response to maternal stress. Offspring exposed to prenatal maternal stress alone have smaller hippocampal volumes than animals in the other groups. These results are in agreement with other research which shows that depressed patients often have a smaller hippocampal volumes [33]. This might be the result of suppressed neurogenesis or altered cellular turnover rates [33]. This decrease in hippocampal volume in stressed offspring is probably due to increases in cortisol concentration in the mother, which also reach the fetus via the placenta [66]. These glucocorticoids may directly influence hippocampal volume. However, the mechanisms of the effect of glucocorticoids on hippocampal volume are still not known and has to be investigated. Hippocampal volume losses translate into disrupted function, as indicated by the cognitive impairments, which are symptoms of major depression [67]. Altered hippocampal function can also have an influence on the activity of other brain areas, such

as the prefrontal cortex and the amygdale, which play a key role in the regulation of emotion [67]. In addition, a disturbed hippocampal function can further contribute to HPA axis dysregulation, since the hippocampus is important in the negative feedback control of the HPA axis [67].

#### ***4.5 Postnatal fluoxetine exposure increases hippocampal cell proliferation in adolescent offspring***

Findings from the present study demonstrate that adolescent offspring exposed to maternal stress and fluoxetine have a significantly decreased density of cell proliferation in the GCL/SGZ of the hippocampus compared to controls. However there was a tendency that offspring exposed to maternal stress and fluoxetine have a higher density of cell proliferation in the GCL/SGZ of the hippocampus than offspring exposed to either maternal stress or fluoxetine. These results indicate that prenatal maternal stress decreases hippocampal cell proliferation, and maternal fluoxetine treatment may partially reverse this reduction in cell proliferation. These findings are in agreement with research in adult rats on the effects of fluoxetine [34, 68, 69]. It has been established that chronic antidepressant treatment significantly upregulates neurogenesis in the dentate gyrus of the hippocampus. Administration of several classes of antidepressants was found to increase BrdU-labeled cell number, which indicates that this is a common and selective action of antidepressants [34]. Chronic antidepressant treatment reverses the inhibition of neurogenesis induced by corticosterone treatment [68]. However, one study has shown that when mice are exposed to early life stress and chronic exposure to fluoxetine treatment during adulthood, there was no effect of fluoxetine on adult hippocampal neurogenesis [69]. In our study, offspring was already treated with fluoxetine via their mothers in the preweaning period, which can explain the partially increase in hippocampal cell proliferation in offspring exposed to maternal stress and postnatal fluoxetine. The mechanisms responsible for hippocampal neurogenesis are under investigation. It has already been shown that both the cAMP cascade and BDNF are upregulated by antidepressants and play a role in the regulation of neurogenesis [34].

Furthermore, chronic fluoxetine treatment has also sex-specific effects on hippocampal cell proliferation. It has been reported that in male mice, a low dose of fluoxetine produces the largest increase in cell proliferation, whereas high doses of fluoxetine leads to more production of new cells in the hippocampus of adult female mice [70]. Age specific effects are also seen with antidepressants. Fluoxetine treatment only increases cell proliferation in adult male rats but not in adolescent males or female rats at any age or stage in the estrous cycle [35]. The exact pathways by which antidepressants increase cell proliferation has to be investigated yet. It is important to elucidate the mechanisms responsible in the male and in the female by which



antidepressants act, especially with regard to treatment.

Interestingly, exposure to maternal fluoxetine treatment in the absence of maternal stress decreases hippocampal cell proliferation in adolescent offspring. Others have shown that fluoxetine treatment alone leads to an increase of hippocampal cell proliferation in adults [34]. Although this was not what we expected, it can be explained by the fact that antidepressants affect the developing serotonin homeostasis in offspring. It seems that fluoxetine only leads to increases in cell proliferation in offspring exposed to maternal stress. Maternal stress may lead to a decrease in cell proliferation, and probably a dysregulation of the serotonin system, which antidepressants try to counteract. When there is no exposure of offspring to maternal stress, the fluoxetine treatment affects developing systems, which are undisturbed yet, and thereby cause a decrease in cell proliferation. Other research showed that prenatal fluoxetine treatment can lead to a decrease in the serotonin transporter levels in the raphe nucleus and subsequent alterations in depressive- and anxiety- related behaviors [28]. Probably, there is an effect of fluoxetine exposure on the developing serotonin system of the offspring, which causes a decrease in serotonin transporter density. This can have a direct effect on cell proliferation in the hippocampus. Although, more research is needed to identify the mechanisms responsible for the decrease in hippocampal cell proliferation after exposure to postnatal fluoxetine in offspring.

Other research has also investigated the effects of prenatal stress on cell proliferation in the early developing brain. This study revealed that prenatal maternal stress induces a decrease in the density of cell proliferation in the nucleus accumbens and hippocampus [37]. The mechanisms by which prenatal stress affect hippocampal development are still unclear. A possible explanation is that the HPA axis is activated by prenatal stress. It has been shown that excessive maternal stress hormones are secreted into the placental blood and as a result are able to reach the fetal brain. This can result in dysregulation of the HPA axis [37]. It has been recently shown that the corticosterone released during stress, as well as synthetic glucocorticoid, have inhibitory effects on neonatal cell proliferation and adult neurogenesis in the dentate gyrus [37]. For example, excess of maternal glucocorticoids may interact with glucocorticoid receptors in the hippocampus and impair the early hippocampal development.

Some studies also report sex-specific effects of prenatal or early life stress on offspring. It has been reported that only female offspring (75 days of age) exposed to prenatal maternal stress, and not the males, had fewer hippocampal granule cells than their non-prenatally stressed counterparts [71]. Also, 24 hours of maternal deprivation in the early postpartum period seems to affect hippocampal structural plasticity in a sex-dependent manner. Neurogenesis was elevated in males, but decreased in females after maternal deprivation [72]. This indicates that the early development of the female brain

is more vulnerable to prenatal stress, probably because of the involvement of female sex hormones in the development of the brain. In addition, research showed that there is an age-dependent effect of prenatal stress on hippocampal cell proliferation in female rats [38]. However we have not shown sex-dependent effects of these behaviors in the present study. This may be due to the fact that at the time point the offspring was sacrificed, they did not reach puberty yet, and therefore did not exert sex differences in cell proliferation. More research is needed to identify the mechanisms which are responsible for the effects of stress on brain development in male and female offspring.

#### ***4.6 There is a correlation between the percentage of time spent in arched-back nursing and hippocampal cell proliferation***

In this study, changes in arched-back nursing, which is an active state of nursing, seem to affect the level of cell proliferation in the hippocampus, via changes in Ki67-ir cell densities. An increase in the percentage of time the dams spent arched-back nursing was correlated with lower cell proliferation in the GCL/SGZ of the hippocampus. This result indicates that pup-directed care is associated with hippocampal cell proliferation in adolescent offspring. Other research has shown that maternal care during the first week of postnatal life affects hippocampal development and function [73]. With regards to hippocampal cell proliferation, adult offspring of mothers which exhibited a higher frequency of pup licking/grooming in the first postnatal week showed increased hippocampal synaptic density and enhanced spatial learning and memory [73]. Increases in pup licking/grooming were related with increases in neuronal survival in the GCL/SGZ of the dentate gyrus, but not cell proliferation in adult male rats [74]. Prolonged periods of mother-infant separation in the rat during the first week of life have also been correlated with an increase in apoptosis, decreased neurotrophic factor expression and reduced mossy fiber density in adulthood [74]. Others have also demonstrated that decreases in licking/grooming of pups are associated with shorter dendritic branch length and lower spine density in CA1 cells in adult offspring [75]. In this study, we did not find an effect of licking/grooming and hippocampal cell proliferation in adolescent offspring, but this may be due to differences in the observation and quantification as well as differences in strain and the age of the offspring. In addition there may be effects of maternal care on levels of new cell survival. More research is needed to elucidate the exact mechanisms by which maternal care affect hippocampal cell proliferation and/or survival.

## **5. Conclusions**

Findings from the present study provide important evidence that maternal stress and postnatal fluoxetine exposure has profound effects on offspring development. Maternal stress and fluoxetine affect both behavioral outcomes, such as depressive-like behavior, and neurobiological correlates, such as hippocampal cell proliferation. Here we also demonstrate that there is an association between levels of maternal care and hippocampal cell proliferation. Future work should aim to determine the effects of maternal stress and postnatal fluoxetine exposure on hippocampal neurogenesis in adolescent offspring. It is also important to measure glucocorticoid receptor levels in the hippocampus. In addition, further research is needed to determine how postnatal fluoxetine affects the serotonin system in offspring, for example by quantifying the serotonin transporter density in the hippocampus and serotonin levels.

## 6. References

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**Effects of maternal stress and postnatal fluoxetine exposure on affective-like behaviors and hippocampal cell proliferation in adolescent rats**

Richting: **master in de biomedische wetenschappen-klinische moleculaire wetenschappen**

Jaar: **2010**

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