Depression during pregnancy: The dilemma

The effects of SSRIs during pregnancy on social behavior of mothers and their offspring

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Abstract

Depressive symptoms during pregnancy may have a tremendous impact on the developing child. Unfortunately, this is true for antidepressant treatment as well. So far, it is unclear whether antidepressants increase the risks for the offspring. We therefore studied the effects of antenatal depression, antidepressant treatment, and their combination on social behavior in mothers and their offspring. We maternally separated heterozygous serotonin transporter knockout ($SERT^{+/-}$) rats for 6 hours a day from postnatal day (PND)2 to 15 and used this as a model for antenatal depression (F1). Control $SERT^{+/-}$ rats were handled for 15 minutes from PND2 to 15. Once pregnant, depressed and control mothers (F1) were daily treated with 10 mg/kg fluoxetine (a selective serotonin reuptake inhibitor) or placebo until pups were weaned. Before treatment mothers were tested for sociability in the three-chamber test and this was repeated after treatment. After weaning of the pups (F2), mothers were sacrificed and BDNF long 3'UTR expression was measured in the prefrontal cortex. Offspring (F2) was tested for social play behavior at juvenile age and social interaction and aggressive behavior (only males) during adulthood. Our results showed that fluoxetine decreased maternal weight gain during pregnancy, but did not alter litter sizes. However, the survival rate of the offspring (F2) exposed to fluoxetine was about 50% during the first postnatal week. No differences were found in sociability of depressed mothers (F1) before and after drug treatment. Also, fluoxetine treatment did not alter BDNF long 3'UTR expression in depressed mothers (F1) compared to controls. Offspring (F2) of depressed mothers showed an increase in social behavior in the social play and social interaction test. This effect was reduced by fluoxetine exposure. Overall we show that antenatal depression increases social behavior of the offspring (F2) and that fluoxetine reverses this effect. This may be of translational value as only depressed pregnant women take antidepressants. More research is necessary to give more insight in the effects of antidepressants on top of antenatal depression.

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Introduction

Although pregnancy is often portrayed as a time of great joy, that's not the reality for all women. Depressive symptoms during pregnancy is not uncommon, in fact 20% of women experience some depressive symptoms during any time of their pregnancy (Ryan et al., 2005). The number of women who suffer from major depression during pregnancy is estimated to be 4-8% (Melville et al., 2010; Kim et al., 2015). According to the DSM V, this disorder is characterized by a depressed mood or a loss of interest or pleasure in daily activities for more than two weeks. Depression is accompanied by impaired social, occupational, and educational functioning. Untreated antenatal depression may have a tremendous effect on the developing child. One of the underlying mechanisms that contributes to depression is the Hypothalamic-Pituitary-Adrenal (HPA) Axis, which is involved in stress regulation. Continued activation of the HPA-Axis in depressed patients causes an elevated stress response and increased cortisol levels (Field et al., 2004). Forty percent of the cortisol passes through the placenta (Gitau et al., 1998), consequently increased cortisol levels are found in the urine and saliva of the infants of depressed mothers (Kaplan et al., 2008). Fetal exposure to increased maternal stress levels impacts the developing child. For example, high levels of maternal cortisol is associated with a reduced neurological development (Ellman et al., 2008) and altered cortisol responses of the unborn child to a stressor (Davis et al., 2011). Furthermore, antenatal depression has also been linked to reduced fetal growth (El Marroun et al., 2012; Henrichs et al., 2010), altered cardiovascular responses to stress (Fan et al., 2015), a higher chance of developing depression during adolescence (Plant et al., 2015) and adulthood (Pearson et al., 2013), or developing other psychopathologies (Pawlby et al., 2011). Thus, depression during pregnancy can negatively influence the unborn child on both physiological and behavioral levels. Treatment with antidepressants may relieve the symptoms of the depression of the mother and could help in reducing the impact on the unborn child.

Nowadays, a considerable number of women is treated with antidepressants during pregnancy. In Europe this concerns 2-3% of the pregnant women (Kieler et al., 2012; El Marroun et al., 2012), while in the U.S. the occurrence is as high as up to 13% (Cooper et al., 2007; Hayes et al., 2012). The most prescribed antidepressants are selective serotonin reuptake inhibitors (SSRIs), because of their good efficacy, few side effects and therapeutic safety (Barbey & Roose, 1998). SSRIs block the serotonin transporter and thereby inhibit the reuptake of the neurotransmitter serotonin into the presynaptic cell. As a result, the extracellular serotonin levels are increased. Although SSRIs are considered safe for antenatal use (Gentile, 2005), it has been reported that the use of SSRIs during pregnancy may negatively influence the development of the unborn child. SSRIs can cross the placenta and are found in the amniotic fluid (Hostetter et al., 2000; Loughhead et al., 2006), affecting therefore not only the mother but also the developing child. During brain development serotonin acts as a neurotrophic factor, regulating cell division, differentiation, migration, growth cone elongation, dendritic pruning, myelination, and synaptogenesis (Gaspar et al.,

2003). Thus, changes in the serotonin levels during neurodevelopment, for instance by administration of SSRIs by the mother during pregnancy, potentially affect a number of processes in the offspring. Indeed, literature shows a number of side effects in the offspring due to prenatal SSRI exposure. First of all, SSRI exposure during pregnancy has been associated with attenuated basal cortisol levels in neonates (Brennan et al., 2008; Pawluski et al., 2012), and differential cortisol levels in 3 month old infants in response to a stressor (Oberlander et al., 2008). Also, the neonatal heart rate response to an acute noxious event is attenuated (Oberlander et al., 2002). Furthermore, several behavioral changes have been reported, such as increased internalizing behaviors of 3-year-old children (Oberlander et al., 2010), increased externalizing behaviors in 4-year-old children (Oberlander et al., 2007), and disrupted sleep patterns in newborns (Zeskind & Stephens, 2004). Recently, there has been much interest in the link between SSRI treatment and autism spectrum disorders (ASDs). ASD is a neurodevelopmental condition characterized by difficulties in social communication and unusually restricted, repetitive behavior and interests. The available literature shows an association between the prenatal use of SSRIs and the increased risk of ASDs in the child (reviewed by Gentile, 2015). It is theorized that this is facilitated by an increase in serotonergic activity during brain development (Whitaker-Azmitia, 2005).

Thus, several studies have shown an increased risk for the developing child both during antenatal depression and after prenatal SSRI exposure. However, it is difficult to discern between the effects of the SSRIs and the effects of the depression itself, as healthy mothers do not administer antidepressants. The effects could be due solely to the administration of the SSRIs, or alternatively, the SSRIs are only partially effective and therefore don't eliminate all the adverse effects of the depression thereby adding up to the adverse effects of antenatal depression (reviewed by Olivier et al., 2015). Therefore, in this study we would like to disentangle the effects of maternal depression, maternal antidepressant treatment and their combination on the offspring.

Almost all the pre-clinical experiments that study the effects of antidepressants during pregnancy have been conducted in healthy rats. Since healthy pregnant women do not take antidepressants the translational value of these studies is questionable. To make a valid translational step to humans this study makes use of the maternally separated heterozygous serotonin transporter knock-out rat (MS-SERT$^{+/-}$). The serotonin transporter expression of the SERT$^{+/-}$ rat is decreased with 40-50% (Homberg et al., 2007), and this rat shows depression-like traits, such as increased CRH expression in the prefrontal cortex and increased immobility, especially after early exposure to early life stress (maternal separation) (Olivier, unpublished data). This is similar to humans who carry a short allelic variant of the serotonin transporter promoter (5-HTTLPR S-allele phenotype). When people with this polymorphism are exposed to stressors they have a higher risk to develop major depression (Non et al., 2014; Jirtle & Skinner, 2007). As the findings between the human 5-HTTLPR S-allele phenotype and that of the SERT$^{+/-}$ rat are comparable, this rat is suited as a model for the human 5-HTTLPR-S allele phenotype.

In this study we focused on the effects of antenatal depression, treatment with SSRIs, and their combination on social behavior in both the mothers and their offspring. Moreover, we studied the gene expression of BDNF long 3'UTR in the prefrontal cortex, which has been shown to be affected by maternal separation (Calabrese et al., 2015). We hypothesize a decrease in social behavior of the offspring of healthy rats treated with a SSRI, based on previous research (Olivier et al., 2011). Maternal separation of the mother has an intergenerational effect on depressive-like-behaviors (Schmauss et al., 2014), which might be true for social behavior as well. Little is known about the interaction of a depressive state and treatment with SSRIs during pregnancy. Xiong et al. (2015) showed that an intergenerational effect of maternal separation on anxiety can be reversed by SSRI treatment. Although in the Xiong study the SSRIs were not administered during pregnancy but directly in the offspring, we expect to find similar results.

Methods

Overview experimental approach

Both 'depressed' (maternally separated) and control SERT$^{+/-}$ females were used to breed with wildtype males. The depressed and control females were treated with either a SSRI or a placebo (1% methylcellulose), starting the first day of their pregnancy until weaning of the pups (figure 1). Sociability of the females/mothers was measured before and after the drug treatment to check for the effects of both the depression and the drug treatment on social behavior. After weaning of the pups the mothers were sacrificed and BDNF long 3'UTR gene expression was measured in the prefrontal cortex.

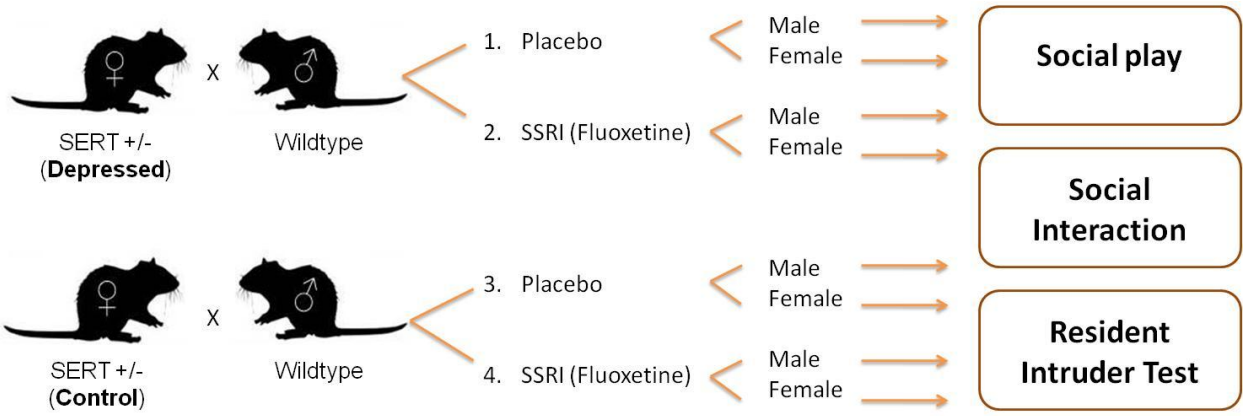

Figure 1 *Schematic overview of the experimental approach. Depressed (maternally separated) or control mothers will be treated with either a SSRI or a placebo, leading to 4 different experimental groups. Both the male and female and SERT $^{+/+}$ (wildtype) and SERT$^{+/-}$ (heterozygous) offspring of these groups will be tested on social behavior.*

The male and female SERT$^{+/-}$ and SERT $^{+/+}$ offspring of these mothers was tested for social behavior in several behavioral tests. They were subjected to a juvenile social play test, social

interaction test, and to a resident intruder test (only males), at the age of 4, 10 and 14-16 weeks respectively. The body mass of the offspring was measured weekly.

Animal model and housing

As a model for antenatal depression the litters of twenty female heterozygous serotonin transporter knockout ($SERT^{+/-}$) rats were maternally separated for 6 hours per day from postnatal day (PND)2 to 15. As control, ten $SERT^{+/-}$ rats were daily handled for 15 minutes from PND2 to 15.

$SERT^{+/-}$ offspring (F1 generation) was used as depressed (maternally separated; MS-$SERT^{+/-}$) and control (CTR-$SERT^{+/-}$) females for breeding. The females were group housed (2-5) with their siblings of the same sex. At the age of approximately 10-11 weeks, these F1 females were bred with wildtype males, single housed until their pups (F2 generation) were weaned. After weaning the offspring (F2) was group housed with their same-sex siblings in group sizes varying from 2 to 4.

All animals were housed under standard conditions (21±2°C, 50±5% humidity) in standard macrolon type 4 cages containing wooden shavings bedding, nesting material and wooden gnawing sticks. The rats were housed on a 12:12 hour light/dark cycle, with lights off at 11 a.m. during the first sociability test and social play test, and light of at 10 a.m. during the second sociability test, social interaction test and resident intruder test. The animals had at libitum access to water and RHM-B chow (ABDiets, Woerden, The Netherlands).

Breeding an drug treatment

When the depressed and control females (F1) reached the age of 10-11 weeks we started the breeding protocol. Daily around 11 a.m. the estrous stage of the rats was assessed with an impedance checker (Impedance Checker MK-10-B, Muromachi; Ramos et al., 2001). When the estrous stage was reached the female was placed with a $SERT^{+/+}$ (wildtype) male. The next day the male rat was removed and this day was considered gestational day 1 (G1).

From G1, mothers were daily treated with 10 mg/kg fluoxetine (a SSRI) or placebo until pups were weaned, a total drug treatment period of 6 weeks. As a placebo, a 1% methylcellulose solution was used, which was the constituent of the fluoxetine pills. Before the drug treatment the females were tested for their sociability in a Three Chamber test. The females were ranked on sociability and then sequentially assigned to the fluoxetine or placebo group, in order to prevent a social bias in the groups. During the drug treatment period, the body mass of the females was measured daily. When the pups (F2) were born, the litter size was assessed and pups were weighed on postnatal day (PND)7, 14, 21, 28, 35, 42, 49, 56, 63, and 70. At PND21 pups were weaned and ear cuts were taken from the pups to determine their genotype. After weaning of the pups (F2) the mothers (F1) were again subjected to a sociability test and subsequently decapitated and several brain areas were collected.

Genotyping

The genotype of the animals was assessed via DNA isolation and qPCR. DNA was isolated from ear tissue via the protocol of Cuppen (2010). Subsequenlty the DNA samples were processed for quantitative real time polymerase chain reaction (qPCR) to assess genotype. Each sample was treated with TaqMan® Genotyping mastermix. Primers were used as a starting point for the PCR for the conding-strand (forward) and the template-stand (reverse). As a probe we used opposite DNA strands of the SERT gene and used 6-FAM and VIC dye-labels (table 1). DNA was anlysed by a 7500 fast real time PCR system (Applied bio systems®). Thermal cycling was initiated with a 10 minute incubation of 95°C. After this initial step, 40 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 92°C for 15 seconds. Finally the plate was incubated for 1 minute at 60°C.

Genotype	Forward Primer	Reverse Primer	Probe
Sert +/+	5'-GCACGAACTCCTGGAACACT-3'	5'-AGCGTCCAGGTGATGTTGT-3'	6FAM- AGTTGGTGCAGTTGC-MGBNFQ
Sert -/-	5'-GCACGAACTCCTGGAACACT-3'	5'-GCACGAACTCCTGGAACACT-3'	VIC-AGTAGTTGGTTCAGTTGC-MGBNFQ

Table 1 *Sequences of forward and reverse primers used in qPCR analysis for genotyping.*

Gene expression

Gene expression of BDNF long 3'UTR was measured in the prefrontal cortex. RNA was isolated by guanidium isothicyanate (inactivation of RNases) with the use of TRIzol, and acidic chloroform was used for partitioning of RNA into aqueous supernatant for separation. Following RNA extraction, the RNA samples were processed for quantitative real time polymerase chain reaction (qPCR) to assess long 3'-UTR BDNF. RNA was anlysed by a TaqMan qRT PCR instument (CFX384 real time system, Bio-Rad Laboratories). Samples were run in 96 well formats in triplicate as multiplexed reactions with two normalizing internal controls. Thermal cycling was initiated with an incubation at 95°C for 10 min (RNA retrotranscription). Subsequently 40 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 92°C for 15 seconds followed by 1 minute annealing at 58°C. The log2 mean normalized expression was calculated per female. Subsequently the difference between the average gene expression of the control group (control handled and methylcellulose treated), and the experimental groups was calculated and expressed as the fold change.

Behavioral tests

Sociability – Three Chamber test: Social interest of the mothers (F1) was assessed before and after the drug treatment, with the use of a Three Chamber set-up (figure 2). The interest of the animal for a social stimulus (a conspecific placed in one of the wired cages) versus a neutral stimulus (an empty wired cage) was measured, as described previously (Kaidanovich et al., 2011). The rats were allowed to habituate for 5 minutes in the center chamber. Subsequently an age- and sex-matched novel rat was placed in one of the wired cages. The

doors dividing the center and outer chambers were removed and the subject rat was allowed to move freely through the apparatus for ten minutes. Time spent in the chamber where the wired cage contained the conspecific (novel) rat was taken as a measure for sociability. Testing before and after drug treatment both took place during the dark phase (between 10 a.m. and 2 p.m.). The test before drug treatment was conducted in the dark, while the test after drug treatment was conducted under dim light conditions. Before the first but not the second test, the animals were habituated in their own cage in the experimental room for 30 minutes. A video camera was mounted above the apparatus, and EthoVision® XT (version 10) videotracking was used to analyze time spent in each of the chambers. Shortly after the test a smear was taken from each subject, to determine in which phase of the estrous cycle the animals were in.

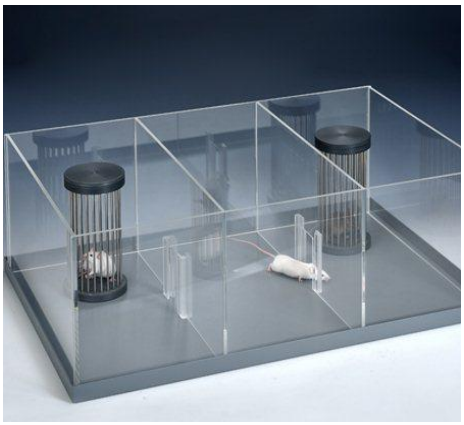

Figure 2 *Three Chamber apparatus. The cage (120 x 80 x 40 cm) contains a center chamber and two outer chambers (40 x 80 x40 cm). The outer chambers include wired cages for a stranger rat.*

Social play: Social play behavior of the offspring (F2) was assessed when the rats were 28-35 days of age, as described previously (Homberg et al., 2007; Olivier et al., 2011). The animals were tested in a plastic instrumented observation cage (45 x 30 x 50 cm) with approximately 3 cm of wood shavings covering the floor. At the end of the light phase (between 9 and 11 a.m.) the animals were singly habituated to the test cage for 5 minutes and afterwards socially isolated for 3.5 hours to induce a maximal increase in the amount of social play (Niesink & Van Ree 1989). After the isolation period, sex-, age-, and treatment-matched pairs were placed in the test cage and tested for social play for 15 minutes. Testing took place during the dark phase, between 12 p.m. and 3 p.m. The test pairs had no previous common social experience. Behavior of the animals was recorded with a PhenoTyper® topping including a camera on top of the cage, and analyzed afterwards with Observer® XT version 10. Duration and frequencies of the following behaviors were scored: (1) pouncing: play soliciting by nosing the partner's nape; (2) pinning: one of the animals is lying with its dorsal surface on the floor with the other animal standing over it; (3) boxing/wrestling: the rats are facing each other in a vertical position and struggling using their forepaws; (4) following/chasing: moving towards the test partner, who moves away; (5) social exploration: sniffing or licking any body part of the test partner. The behaviors were assessed per pair of animals.

Social interaction: When the offspring was 10-11 weeks (PND70-PN77) of age, the same pairs who were used in the social play test were tested again for social interaction. Except for the 15 minutes during the social play test the test pairs had no previous common social

experience. A wooden cage (85 x 55 x 40 cm) with plastic sliding doors covering the whole front of the cage was used as a test cage. The floor of the cage was covered with wood shavings. Prior to the test the rats were socially isolated for 48 hours. On the second and first day prior to the test the animals were singly habituated in the test cage for 20 minutes. On the test day the rats were placed in pairs in the test cage and their behavior was recorded for 15 minutes, with a camera placed in front of the cage, and analyzed afterwards with Observer® XT. Duration and frequencies of behaviors mentioned at the social play test were scored, however, social exploration was divided into: (1) social sniffing: sniffing any body part of the test partner ; (2) social grooming: grooming or licking the any body part of the test partner ; (3) passive contact: lying or sitting with body's in contact but without interacting with each other. The behaviors were assessed per pair of animals. Shortly after the test a smear was taken from each female subject, to determine in which phase of the estrous cycle they were in. Habituation and testing took place during the dark phase between 11 a.m. and 3 p.m., the test was performed under dim light conditions.

Resident intruder test: The male offspring was tested for aggression in the resident intruder test. Due to practical reasons the age of the $SERT^{+/+}$ (wildtype) male offspring (F2) was around 14 weeks during the test, while the age of the $SERT^{+/-}$ (heterozygous) male offspring was around 16 weeks. The test was assessed as described previously (Koolhaas et al., 2013). The male subjects were housed in large observation cages, described in the social interaction test, each with a sterilized female. The cages contained wooden shavings on the floor, 2 wooden chewing sticks, a tube for hiding, a water bottle, and a hand of food. After one week of social housing with a female, the baseline level of offensive behavior was measured on three consecutive days during a 10 minute confrontation with an unfamiliar male conspecific (intruder) in the home territory of the experimental (resident) rat. At least one hour prior to the confrontation the female partner of the resident was temporarily moved to another room. Also, the tube was removed, to prevent the rats from hiding in it. On day one to three, when the resident attacked the intruder, the attack latency was noted and the confrontation was stopped (even when the 10 minutes were not over). On the fourth day, the residents were challenged with an intruder again, and the behavior of the animals was recorded up to 10 minutes after the first attack (up to a maximum recording of 20 minutes). The training and test sessions were performed under dim light conditions during the dark phase between 11 a.m. and 3 p.m., all females were removed at 9.30 a.m., and placed back in the home cage after testing. The intruders were group housed, and each resident was challenged with another intruder on each of the four days. The residents were previously used in the social play and social interaction test, the intruders were naïve to behavioral testing. Behavior was analyzed afterwards with Observer® XT version 10. Duration and frequency of the following behaviors was scored: (1) attack latency; (2) move towards; (3) social exploration; (4) ano-genital sniffing; (5) rearing; (6) lateral threat; (7) upright posture; (8) clinch attack; (9) keep down; (10); chase; (11) non-social exploration; (12) rest or inactivity. The behavioral elements are expressed as percentage of total duration of the confrontation.

A schematic representation of the timeline of the experimental protocol is displayed in figure 3.

Statistical analysis

Body mass of both the mothers (F1) and the offspring (F2) was analyzed with a two-way ANOVA for repeated measures, with maternal treatment and antenatal drug as between subject factors, where appropriate a least significant difference (LSD) post-hoc test was performed. The effect of drug treatment on behavior of the mothers (F1) in the sociability test was analyzed using independent sample *t* tests for the data before the drug treatment. Data before and after the drug treatment was analyzed with a two-way ANOVA for repeated measures, with maternal treatment and antenatal drug as between subject factors, where appropriate data was further analyzed with a LSD post-hoc test or a paired samples *t* test. BDNF long 3'UTR expression (F1) was analyzed with a two-way ANOVA with maternal treatment and antenatal drug as variables. Initial and final litter size was analyzed with an independent sample *t* test. Social play at juvenile age and social interaction at adulthood (F2) were analyzed with an independent sample *t* test if data of only two experimental groups was available. When the data of three experimental groups was available a one-way ANOVA was used, with LDS post-hoc testing if appropriate. The attack latency in the resident intruder test (F2) was analyzed with a one-way ANOVA. Level of significance was set at $p < 0.05$ (n.s. = non-significant). All statistical analyses were performed using the Statistical Package for the Social Sciences version 22.0 for windows (SPSS Inc., Chicago, IL, USA). Error bars represent standard errors of the mean (S.E.M.).

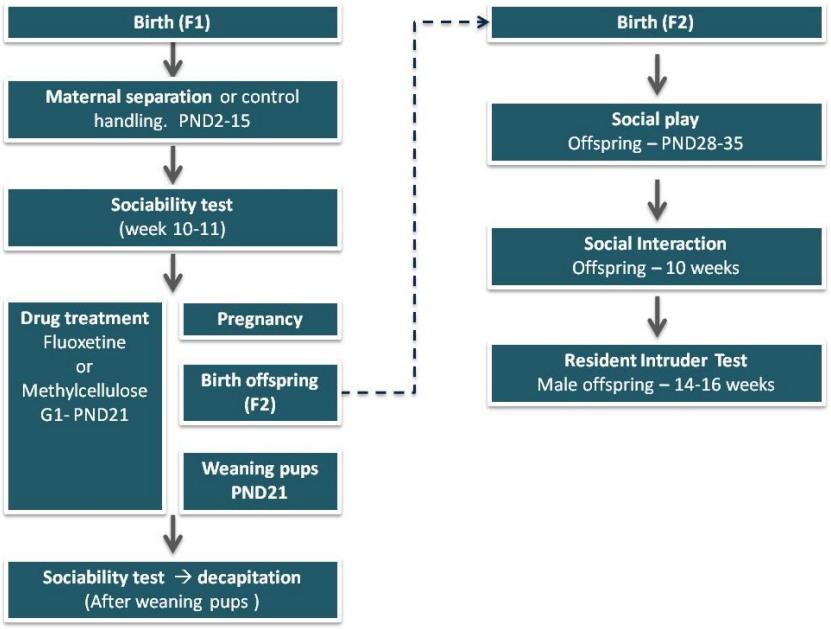

Figure 3 *Timeline. Mothers (F1) were maternally separated PND2-15, and tested for sociability at adult age. Once pregnant mothers were daily treated with fluoxetine or 1% methylcellulose until weaning of the pups. Afterwards, mothers were again measured for. Offspring (F1) was tested for social behavior in the social play test, social interaction, and resident intruder test.*

Results

Of the thirty females (F1) only nineteen actually became pregnant during the day they were paired with a male rat. We have treated both pregnant and non-pregnant rats with fluoxetine or the placebo. Data of females who were not pregnant during the period of drug administration will not be discussed in this report and can be found in the supplementary data. Composition of the different treatment groups (F1) is shown in table 2.

Maternal treatment	Drug treatment	Group	N=
Control handling (C)	Methylcellulose (MC)	C-MC	2
Control handling (C)	Fluoxetine (FLX)	C-FLX	2
Maternal separation (MS)	Methylcellulose (MC)	MS-MC	8
Maternal separation (MS)	Fluoxetine (FLX)	MS-FLX	7

Table 2 *Overview of the various maternal treatment groups (F1) and group sizes.*

Effect of the drug treatment on body mass – F1

Body mass of the mothers (F1) was measured daily during the period of drug treatment. A time x drug interaction was found for body mass. ($F_{(1,41)}$= 5.09; p<0.001). Moreover, a time x maternal treatment x drug interaction was found ($F_{(2,41)}$= 1.569; p<0.05).

Further analysis showed that both the control and maternally separated group treated with fluoxetine had lower body mass than both the control and maternally separated group treated with methylcellulose (figure 4). This effect was evident from the third treatment day. F- and P-values for each treatment day are shown in supplemental 1.

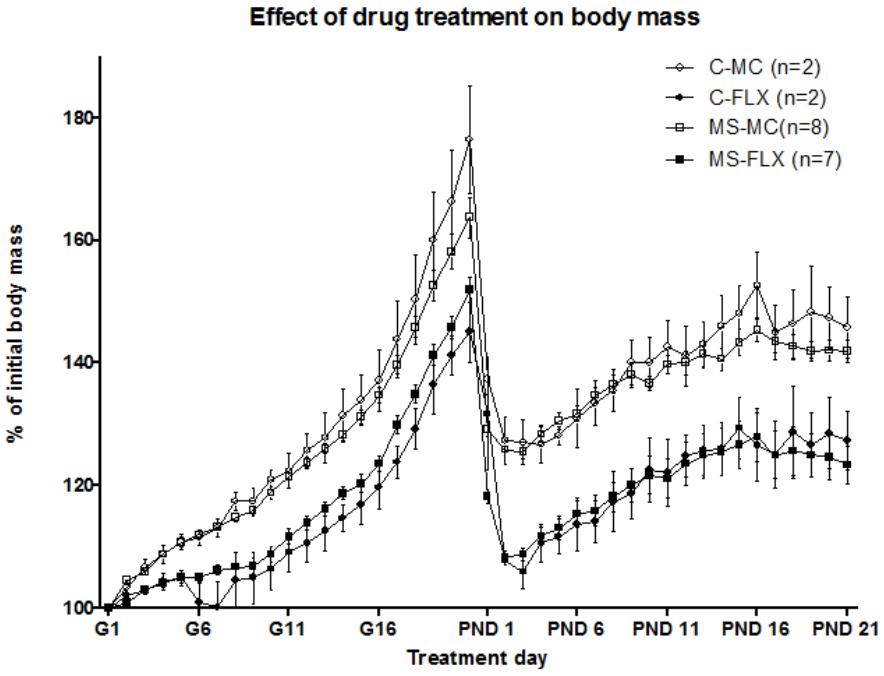

Figure 4 *Effect of fluoxetine on body mass of pregnant females expressed as the percentage of the body mass on the starting day of the treatment. The x-axis represents the treatment (G1= gestational day 1) and subsequently the age of the pups (PND1= post natal day 1). Data are presented as mean ±S.E.M. body mass.*

C=control handled;
MS= maternally separated;
MC=methylcellulose treated;
FLX=fluoxetine treated.

Sociability before and after drug treatment - F1

The females (F1) that were in oestrus during the sociability test did not show significant different sociability than the females that were not in oestrus, both before ($t_{(1,27)}$= 1,79; n.s.) and after ($t_{(1,28)}$= -1.16; n.s.) the drug treatment. Therefore, oestrus phase was not included as a factor for further analysis. Sociability was defined by the time the female spent in the side where the wired cage contained a novel rat (social side). Before the drug treatment there was no significant difference in sociability ($t_{(1,28)}$= -1.64; n.s.) between the maternally separated and the control handled females (figure 5). When the sociability before and after drug treatment was compared, a trend was found in the time x drug interaction ($F_{(1,17)}$= 3.56; p=0.08). One-way ANOVA revealed no differences between the different groups after drug treatment ($F_{(1,17)}$= 0.746; n.s.) (figure 6).

Sociability after maternal separation

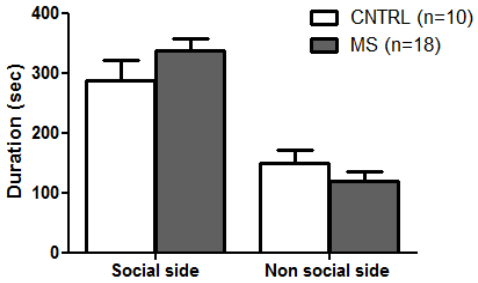

Figure 5 *The effects of maternal separation on sociability. Data are presented as mean ±S.E.M. time spent in the social and non-social side of the Three Chamber apparatus.*

CNTRL: control handled; MS: maternally separated.

Sociability before and after drug treatment

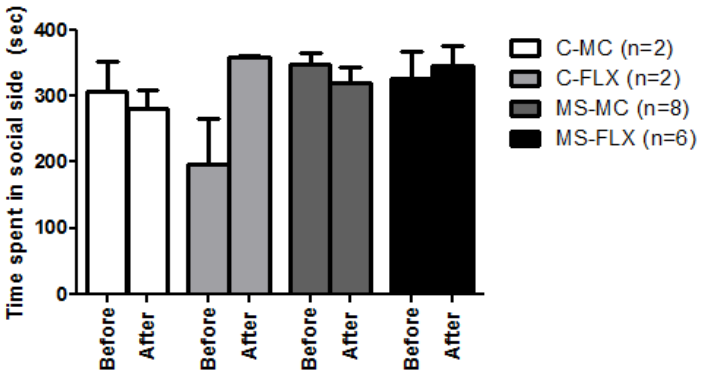

Figure 6 *The effect of fluoxetine treatment during pregnancy in maternally separated and control handled females. Bars represent the time spent in the social side before and after the drug treatment. Data are presented as mean ±S.E.M. time spent in the social side of the Three Chamber apparatus.*

C: control handled; MS: maternally separated; MC: methylcellulose treated; FLX: fluoxetine treated.

The average total distance moved of the females during the sociability test was also assessed. There was no effect of being in oestrus on total distance moved before ($t_{(1,28)}$= -0.00; n.s.) or after ($t_{(1,28)}$= 0.17; n.s.) the drug treatment, therefore the oestrus stage of the females was excluded from further analysis. Before the drug treatment no significant difference was found for total distance moved between the maternally separated and control handled females ($t_{(1,27)}$=-1.20; n.s.) (figure 7). When differences before and after drug treatment were compared, a trend was found for time x maternal treatment x drug interaction ($F_{(2,16)}$ = 13.0; p=0.08). Moreover, after drug treatment, groups significantly

differed for total distance moved ($F_{(1,17)}$= 4.40; p<0.05). Further analysis showed that the control-methylcellulose treated females had a significantly higher total distance moved compared to the control-fluoxetine treated females (p<0.01), the maternally separated-methylcellulose treated females (p=0.01), and the maternally separated-fluoxetine treated females (p< 0.05) (figure 8). Furthermore, after drug treatment, an increase was found for total distance moved in the control-methylcellulose treated group (paired t test $t_{(1,1)}$= -13.85; p<0.05).

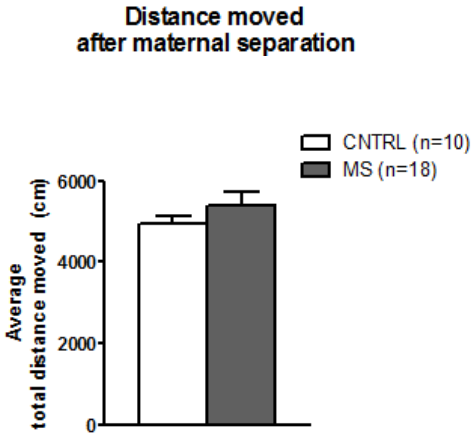

Figure 7 *The effect of maternal separation on total distance moved during a sociability test. Data are presented as mean ±S.E.M. total distance moved (cm).*

CNTRL: control handled; MS: maternally separated

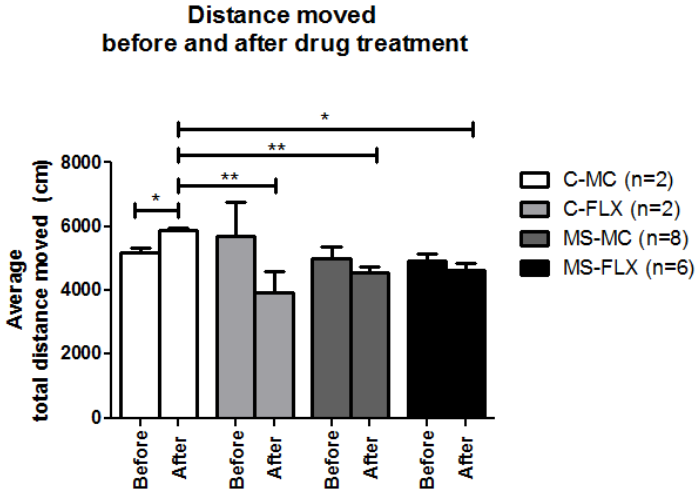

Figure 8 *The effect of fluoxetine treatment during pregnancy in both maternally separated and control females on total distance moved in a sociability test. Data are presented as mean ±S.E.M. total distance moved (cm) before and after drug treatment. *p<0.05,**p<0.01.*

C: control handled; MS: maternally separated; MC: methylcellulose treated; FLX: fluoxetine treated

Gene expression – F1

The BDNF long 3'UTR gene expression in the prefrontal cortex of the different treatment groups (F1) was assessed. Two-way ANOVA showed a trend for treatment x drug interaction in BDNF long 3'UTR gene expression ($F_{(1,17)}$ = 3,57; p=0.08). One-way ANOVA did not reveal differences between the treatment groups ($F_{(1,17)}$= 3.56; n.s.). Although not appropriate, analysis with an independent sample *t* test revealed a significant increase in BDNF long 3'UTR expression in the maternally separated females that were treated with fluoxetine compared to the maternally separated females that were treated with methylcellulose ($t_{(1,13)}$= 2,34; p>0.05) (figure 9; table 3).

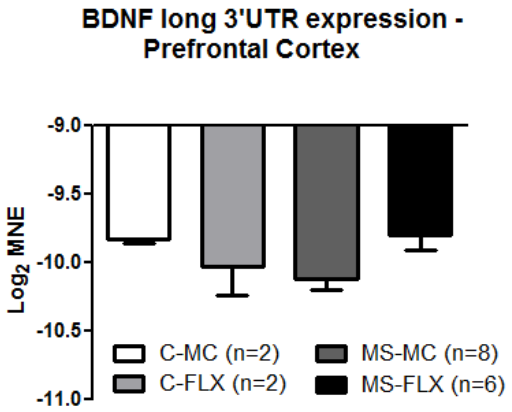

Figure 9 *The effect of maternal separation and/or fluoxetine on BDNF long 3'UTR gene expression in the prefrontal cortex . Data are presented as mean ±S.E.M. log_2 mean normalized expression (MNE).*

C: control handled; MS: maternally separated; MC: methylcellulose treated; FLX: fluoxetine treated.

Treatment group	Log2 fold change
C-FLX	-0.21
MS-MC	-0.29
MS-FLX	0.02

Table 3 *Log2 fold change of different treatment groups compared to the control handled and methylcellulose treated group. A negative value indicates a reduction in expression levels.*

C: control handled; MS: maternally separated; MC: methylcellulose treated; FLX: fluoxetine treated.

Survival chance of the offspring – F2

There was no significant difference in the litter sizes (F2) of the different treatment groups at time of birth ($t_{(1,17)}$=-0.22; n.s.) (figure 10). However, regardless of the maternal treatment (maternal separation or control handling), the survival chance of the offspring of mothers

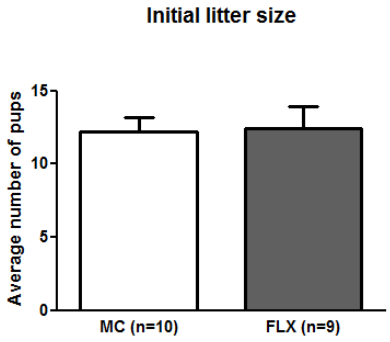

Figure 10 *Effect of fluoxetine exposure during pregnancy on the litter size at time of birth. Data are presented as mean ±S.E.M. mean of pups per nest.*

MC: methylcellulose treated; FLX: fluoxetine treated.

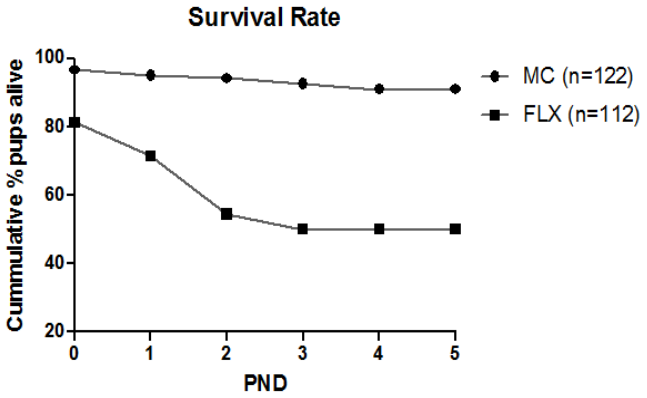

Figure 11 *The cumulative survival rate of pups from mothers treated with fluoxetine (FLX) or methylcellulose (MC). The x-axis represents the age of the pups. All death of pups occurred within 4 days. 91% of the MC pups and 50% of the FLX pups survived.*

MC: methylcellulose treated; FLX: fluoxetine treated.

(F1) treated with fluoxetine was far lower than those of mothers treated with methylcellulose (figure 11). When the initial litter size at time of birth was compared to the final litter size, a time x drug interaction was found ($F_{(1,18)}$ = 9.01; p<0.01). Further analysis showed a significant difference between the final average nest sizes of fluoxetine and methylcellulose treated mothers ($t_{(1,17)}$=3.21; p<0.01.) (figure 12). The differences between final nest sizes of the different treatment groups were less robust when maternal treatment was included as a factor (Supplemental 7).

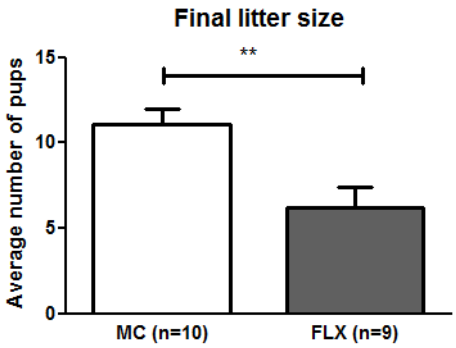

Figure 12 *Effect of fluoxetine exposure during pregnancy on the final litter size. Data are presented as mean ±S.E.M number of pups per nest. **p<0.01*

MC: methylcellulose treated; FLX: fluoxetine treated.

Growth of the offspring – F2

The body mass of both the male and female offspring (F2 generation) was measured weekly, from PND7 until they were ten weeks of age (PND70). No significant differences in body mass were found between the SERT $^{+/+}$ (wildtype) and SERT$^{+/-}$ (heterozygous) offspring in both male and females analyzed for all the different time points. Therefore, for further analysis genotype was excluded as a factor.

For the female offspring a two-way ANOVA for repeated measurements showed a time x maternal treatment interaction ($F_{(1,77)}$ = 3.58; p=0.001) as well as a time x drug interaction ($F_{(1,77)}$ = 4.07; p<0.001). Further analysis with a one-way ANOVA revealed significant differences on PND7 ($F_{(1,77)}$ = 2.99; p<0.05), PND14 ($F_{(1,77)}$ = 5.96; p=0.01), PND21 ($F_{(1,77)}$ = 5.05; p<0.01), and PND28 ($F_{(1,77)}$ = 11.64; p<0.001). Post hoc LSD testing showed that on PND7 the body mass of the pups from the control handled and methylcellulose treated mothers was higher than of the pups from the maternally separated mothers treated with fluoxetine or methylcellulose. On PND14, PND21, and PND28 the body mass of the pups from maternally separated and methylcellulose treated mothers was significantly lower than that of the pups of the other treatment groups (figure 13). The corresponding p-values can be found in supplemental 8.

For the male offspring a two-way ANOVA for repeated measures revealed a time x maternal treatment interaction ($F_{(1,75)}$ = 9.97; p<0.001) as well as an time x drug interaction ($F_{(1,75)}$ = 4.33; p<0.001). Moreover, a time x maternal treatment x drug interaction was found ($F_{(1,75)}$ = 4.44; p<0.001). Further analysis with a one-way ANOVA showed a difference between the treatment groups on PND7 ($F_{(1,78)}$ = 4.82; p<0.01), PND14 ($F_{(1,78)}$ = 7.71; p<0.001), PND21 ($F_{(1,78)}$ = 6.89; p<0.001), PND28 ($F_{(1,78)}$ = 21.83; p<0.001), PND35 ($F_{(1,78)}$ = 5.75; p=0.01), PND49 ($F_{(1,78)}$ = 6.91; p<0.001), and PND56 ($F_{(1,78)}$ = 3.58; p<0.05). Post hoc testing with LSD revealed that on PND7 the pups from control handled and fluoxetine treated mothers had the lowest body mass compared to the pups of other groups. Furthermore, on PND7 the body mass of methylcellulose exposed pups was lower if the mothers were maternally separated compared to control handled. On PND14 and PND21 the pups from maternally separated and fluoxetine treated mothers had higher body mass than the other treatment groups. Moreover, methylcellulose exposed pups from maternally separated mothers had lower body mass than other treatment groups on PND28, PND35, PND49 and PND56 (figure 14). The corresponding p-values can be found in supplemental 8.

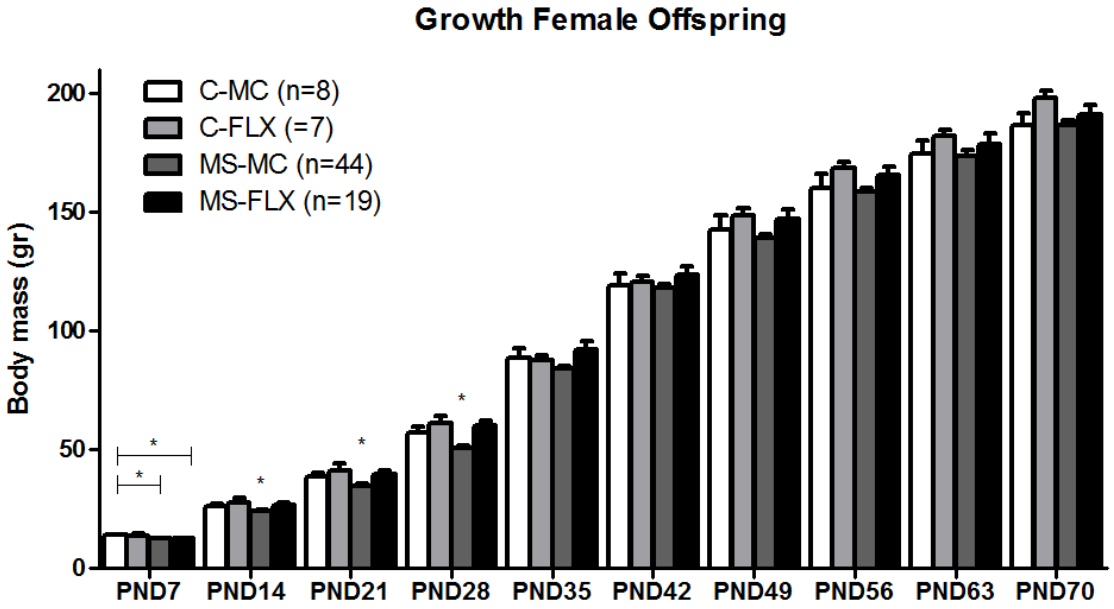

Figure 13 *The effect of maternal separation and/or fluoxetine treatment of the mother (F1) on the body mass of the female offspring (F2) on PND7-PND70. Data are presented as mean ±S.E.M. body mass.*p<0.05.*

C: control handled; MS: maternally separated; MC: methylcellulose treated; FLX: fluoxetine treated.

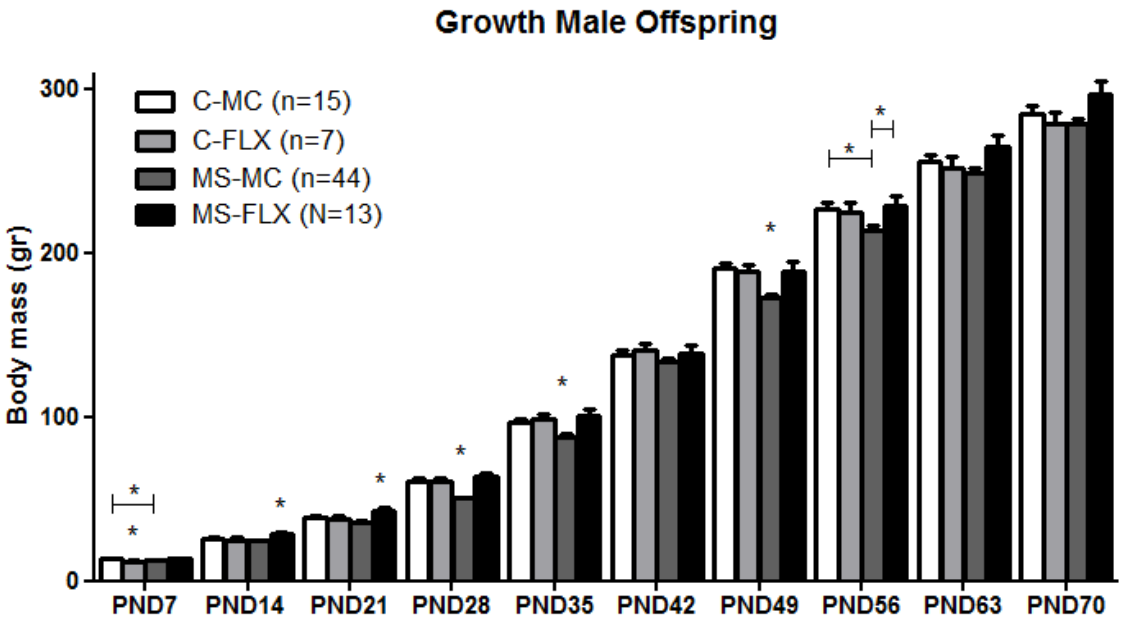

Figure 14 *The effect of maternal separation and/or fluoxetine treatment of the mother (F1) on the body mass of the male offspring (F2) on PND7-70. Data are presented as mean ±S.E.M. body mass. *p<0.05.*

C: control handled; MS: maternally separated; MC: methylcellulose treated; FLX: fluoxetine treated.

Social play behavior of the offspring – F2

Due to the unexpected low survival rate of the offspring, we were not able to create sufficient group sizes for each of the experimental groups (F2). Especially since for the social play and social interaction test age-, sex- and treatment-matched pairs had to be formed. The group sizes for the different treatment groups are displayed in table 4. Only group sizes of two or more pairs have been taken into account for analysis of the social play and social interaction test.

Treatment group (F2)	Males		Females	
	Wildtype	**Heterozygous**	**Wildtype**	**Heterozygous**
Control-Methylcellulose	**3**	**2**	**2**	1
Control-Fluoxetine	0	0	0	0
MS-Methylcellulose	**8**	**8**	**8**	**8**
MS-Fluoxetine	1	**3**	**2**	**2**

Table 4 *Group sizes of the different treatment groups for male and female, wildtype (SERT $^{+/+}$) and heterozygous (SERT$^{+/-}$) offspring (F2). Treatment groups represent offspring from mothers that were control handled (Control) or maternally separated (MS), subsequently the offspring was prenatally exposed to either fluoxetine or methylcellulose. Group size represents the number of age-, sex-, and treatment-matched pairs that were in that specific experimental group.*

For the wildtype male offspring only the methylcellulose exposed offspring from control and maternally separated mothers could be compared. No effect of maternal separation was found for any of the behaviors in the social play test (supplemental 9).

With regard to the heterozygous male offspring methylcellulose exposed offspring from control and maternally separated mothers and fluoxetine exposed offspring from maternally separated mothers were compared. The groups did significantly differ in the time spent on social exploration ($F_{(1,12)}$=8.67; p<0.01). Further analysis showed that the methylcellulose exposed offspring from maternally separated mothers spent more time on social exploration than both the methylcellulose exposed offspring from control mothers (p<0.01), and the fluoxetine exposed offspring from maternally separated mothers (p<0.05) (figure 15). For the others behaviors during social play no effect was found (supplemental 10).

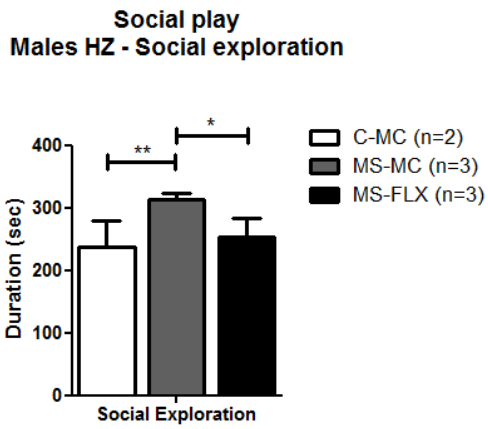

Figure 15 *The effect of maternal separation of the mother and/or prenatal fluoxetine exposure on social exploration during social play in male heterozygous offspring Data are presented as mean ±S.E.M duration spent on social exploration. *p<0.05,** P<0.01.*

C: control handled; MS: maternally separated; MC: methylcellulose treated; FLX: fluoxetine treated.

For the heterozygous females only the fluoxetine or methylcellulose exposed offspring from maternally separated mothers were compared. Fluoxetine exposure led to an increase in time spent on pinning ($t_{(1,9)}$=-4.66; p<0.01) (figure 16). Non-significant effects on other behaviors can be found in supplemental 11.

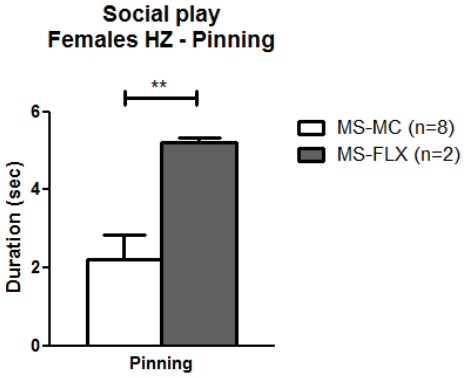

Figure 16 *The effect of treatment on pinning during social play in female heterozygous offspring (SERT $^{+/-}$). Data are presented as mean ±S.E.M. time spent on pinning.** p<0.01.*

C: Control handled; MS: Maternally separated; MC: methylcellulose treated; FLX: fluoxetine treated.

Regarding the wildtype female offspring, methylcellulose exposed offspring from control and maternally separated mothers, and fluoxetine exposed offspring from maternally separated mothers were compared. The groups did significantly differ on frequencies of social exploration ($F_{(1,11)}$= 4.46; p<0.05), following ($F_{(1,11)}$= 4.28; p<0.05), and boxing ($F_{(1,11)}$= 4.11; p=0.05). Moreover, differences were found on the duration spent on pouncing ($F_{(1,11)}$= 5.40; p<0.05) (figure 17). Further analysis revealed that methylcellulose exposed offspring from maternally separated mothers more frequently

showed social exploration, boxing, and following than both the methylcellulose offspring from maternally separated mothers (all $p<0.05$) and the fluoxetine exposed offspring from maternally separated mothers ($p<0.05$, $p=0.06$, $p=0.06$ respectively). Also, in offspring from maternally separated mothers, duration of pouncing was increased in as a result of fluoxetine exposure ($p<0.05$). Non-significant effects can be found in supplemental 12.

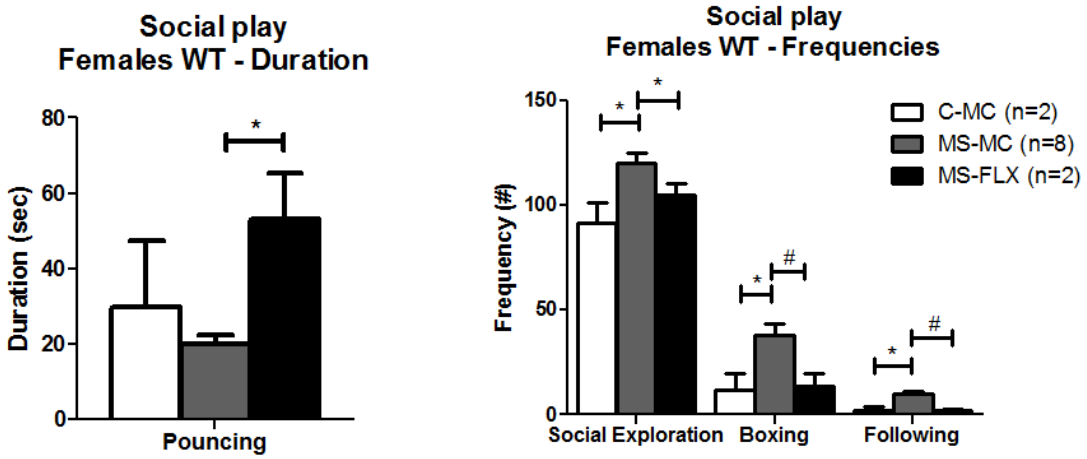

Figure 17 *Effect of maternal separation of the mother and/or fluoxetine exposure on social play in female wildtype offspring. Duration spent on and frequency of a certain behavior are shown on the left and right respectively Data are presented as mean ±S.E.M time or frequency spent on a certain behavior. * p<0.05, #p<0.08*

C: Control handled; MS: Maternally separated; MC: methylcellulose treated; FLX: fluoxetine treated.

Social interaction of the offspring – F2

To date, only the behavior of the male wildtype offspring was analyzed for social interaction at adult age. Only groups with more than two pairs were taken into account for the analysis. Therefore, only the methylcellulose exposed offspring from both maternally separated and control mothers were evaluated.

Maternal separation of the mother led to a significant increase in duration for both pouncing ($t_{(1,11)}=-2.95$; $p<0.01$) and following ($t_{(1,11)}=-2.43$; $p<0.05$). Furthermore, a trend was found for an increase in pouncing frequency ($t_{(1,11)}=-2.05$; $p=0.07$). Also, for the duration of passive contact, a trend was found for a decrease as a result from maternal separation of the mother ($t_{(1,11)}=2.05$; $p=0.07$). Non-significant effects can be found in supplemental 13.

Aggressive behavior of the male offspring – F2

For the resident intruder test, only attack latency (time it took for the resident to attack the intruder) was evaluated so far. Due to practical reasons the wildtype males were tested around the age of 14 weeks, while the heterozygous males were tested around the age of 16

weeks. Therefore, the effect of genotype on the attack latency was not analyzed, since a confounding effect of time could not be excluded. The attack latency was assessed during the three training sessions and during the final test. As with the other behavioral tests, fluoxetine exposed offspring from control mothers is missing. A two-way ANOVA for repeated measurements ANOVA did not reveal any interaction effects for the wildtype or heterozygous offspring. Furthermore, a one-way ANOVA did not show significant results for attack latency on any of the three training days ($F_{(1,33)}$= 0.38; n.s., $F_{(1,33)}$= 1.45; n.s., $F_{(1,33)}$= 0.23; n.s. respectively), the test day ($F_{(1,33)}$= 0.63; n.s.), or the average attack latency of the total four days ($F_{(1,33)}$= 0.67; n.s.) (Supplemental 14).

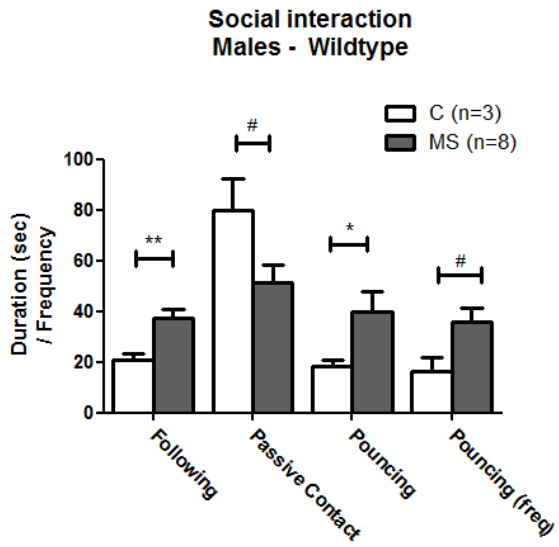

Figure 18 *The effect of maternal separation of the mother on social interaction of the male wildtype methylcellulose exposed offspring at adult age. Data are presented as mean ±S.E.M. time spent on or frequency of a certain behavior.*

*#p<0.08, *p<0.05, ** p<0.005.*

C: Control handled; MS: Maternally separated

Discussion

The aim of the present study was to disentangle the effects of antenatal depression, antenatal treatment with SSRIs, and their combination on mothers and their offspring. We studied the effect on body mass, sociability, and BDNF long3'UTR expression in the prefrontal cortex of the mother (F1). In addition the effect on body mass, juvenile social play, and social interaction and aggression during adulthood in the offspring (F2).

During drug treatment, fluoxetine administration led to a decrease in maternal weight gain during pregnancy. This was expected, as weight loss is a recognized side-effect of fluoxetine (Michelson et al., 1999). Interestingly, we found an over-time interaction of the drug with maternal treatment. The time course for weight changes as a result of fluoxetine treatment was different for maternally separated females than for the controls. However, no differences on body mass were found between maternally separated and control females treated with the same drug at any time point. It would be interesting to assess whether the decrease in weight gain is caused by a reduction in feeding behavior, a metabolic change, or both. And more interestingly, how the depressed animals relate to the control animals in this matter, as depression is associated with both weight loss and weight gain (Wit et al., 2015).

The sociability of the females (F1) was not altered as a result of maternal separation. Similar results were found in a study on the effects of maternal separation in chemokine receptor type 7 deficient mice ($CCR7^{-/-}$) (Harrison et al., 2014). $CCR7^{-/-}$ mice show have higher levels of anxiety, and is an interesting model to investigate the effects of early life stress-induced changes in behavior. They found no effect of maternal separation on sociability. However, when a second stranger mouse was placed in the previously empty wired cage, the maternally separated mice had less preference for social novelty. In our research only sociability was measured, not the preference for social novelty. For future research it might be interesting to see how the animals in our research respond to social novelty. Nevertheless, the influence of maternal separation on social behavior in the $SERT^{+/-}$ rat remains largely unknown. Moreover, fluoxetine treatment didn't result in an altered sociability either. Literature on the effects of SSRIs on sociability are scarce and mainly focused on mice models for autism. The effects of fluoxetine in autism models with impaired sociability show different results. In one of the cases the preference for social novelty was more susceptible to disruption than sociability (Moy et al.., 2013). Another case showed specific effects of fluoxetine; sociability was increased, while preference for social novelty was impaired (Chadman, 2011). The social novel test measures social cognition, which is known to be altered as a result from prenatal SSRI treatment in humans (El Marroun et al., 2014). This indicates the importance of not only measuring sociability, but preference for social novelty as well, to examine the effects of both maternal separation and fluoxetine.

Total distance moved was also assessed in the sociability test to determine the locomotor activity of the animals (F1). The locomotor activity in the 3 Chamber apparatus was not altered as a result of maternal separation. However, after drug treatment the control rats treated with methylcellulose showed a higher distance moved than control rats treated with fluoxetine and maternally separated rats treated with methylcellulose or fluoxetine. Of interest is that the locomotor activity of the maternally separated rats was not altered before drug treatment, while both methylcellulose and fluoxetine treatment led to decrease in locomotor activity in maternally separated rats compared to control rats treated with methylcellulose. The differences in the two tests might be explained by an effect of light conditions. The first three-chamber test was conducted in the dark, while the second test was performed under dim light conditions. The light conditions during the second test may have provoked more fearful reactions. Hence, fear could have been a more important factor during the test after drug treatment than during the test before drug treatment. This is in compliance with data on Wistar rats, who explore less in an open field test as a result from maternal separation (Rana et al., 2015). This theory could also explain the reduced locomotor activity in the control rat treated with fluoxetine, as chronic fluoxetine treatment has been previously classified as anxiogenic after testing with an open field (Gray & Hughes 2015) or elevated plus maze (Silva et al., 1999). However, the control group increased their locomotor activity during the second test compared to the first test. This contradicts the theory that the second test was more fearful. Another possibility is that the rats treated with fluoxetine experienced more handling and injection stress. Injecting these animals was

noticeably harder, perhaps due to aversive effects of SSRIs and therefore these animals were handled more firmly. This may have led to the emergence of differences between control rats and rats treated with fluoxetine.

Although it was not totally statistically substantiated, it appeared that the expression of BDNF long 3'UTR in the prefrontal cortex (PFC) was increased as a result of fluoxetine treatment in the maternally separated rats (F1). BDNF long 3'UTR expression in the dorsomedial PFC, but not in the ventromedial PFC was previously shown to be increased in $SERT^{+/-}$ rats (Calabrese et al., 2015). Maternal separation did not alter these expression levels. Since, we measured the expression in the total PFC it is difficult to compare this with our results. Total BDNF is decreased in major depression (Brunoni et al., 2008), and animal studies show that experimentally induced stress reduced BDNF transcription and synthesis (Bath et al., 2013; Fuchikami et al., 2013). Furthermore, treatment with SSRIs in depressed patients has been shown to increase serum BDNF levels in (Gonul et al., 2005; Aydemir et al., 2005). It would therefore be interesting to see if total BDNF expression in maternally separated rats is decreased and whether or not this can be rescued by fluoxetine treatment.

Thus, we were not able to demonstrate a clear effect of maternal separation on any of the parameters measured in the mothers (F1). Previous research has demonstrated a disturbance in the serotonergic system during early brain development (Ohta et al., 2014). And maternal separation in the $SERT^{+/-}$ rat has shown to lead to depression-like traits in these (Olivier, unpublished data). However, finding a depression marker that predicts the strength of the depression would be assisting in drawing conclusions about the effects of fluoxetine during depression on both the mother and the offspring.

Not all the females (F1) became noticeably pregnant. We've established that this was not due to the fluoxetine treatment, since a similar fraction of methylcellulose and fluoxetine treated females failed to get pregnant. We observed that repeated daily use of males for breeding led to a decrease in the chance of pregnancy. When the males were used for the first time all of them succeeded in impregnating the female. However, the day after impregnating a female only one third of the males succeeded again. After a day of rest, about 45% of the males succeeded, and the success rate increased with each extra day of rest. Four days after mating with a female, all males succeeded to impregnate a second female. Thus, the repeated use of the same males for breeding, with only an intermittent of one or a few days, likely led to failure to impregnate all the females.

The litter size of the mothers treated with fluoxetine was not significantly altered at the time of birth. However, fluoxetine treatment led to a severely reduced survival change for the pups. This was also seen in a study by Noorlander et al. (2008), they treated mice with 0.8 mg/kg fluoxetine from day 8 till 18 of pregnancy. About 80% of the pups prenatally exposed to fluoxetine died within 20 days, and cross-fostering experiments demonstrated that mortality was due to fetal aspects. They observed that the majority died of heart failure, due to dilated cardiomyopathy. Another study (van den Hove et al., 2008) investigated the

effects of 10 mg/kg paroxetine (a SSRI) during the last week of gestation in rats, and also found pre-weaning mortality rates of about 80% in the offspring. A small cross-foster experiment led to the death of all pups, suggesting that SSRIs affect both the mother and the pups. On the other hand, fluoxetine injections (12mg/kg) from gestational day 11 until birth did not lead to higher neonatal mortality (Olivier et al., 2011). But, fluoxetine-treated dams gave birth to fewer pups suggesting prenatal mortality. Own observations during our study suggest reduced postnatal care in dams treated with fluoxetine leading to higher pre-weaning mortality rates. Often, pups were not in the nest, and the umbilical cord and placenta were still attached to the pups until a few days after birth. Recording and identifying the postnatal maternal care, together with a pathological exam of the pups, and possibly cross-fostering experiments should give insight in the high postnatal mortality rates due to fluoxetine. An important consequence of the low survival rate for fluoxetine exposed offspring is the resulting difference in litter sizes between fluoxetine and methylcellulose exposed rats. These confounding factors must be kept in mind in interpreting the differences in behavioral and physiological parameters in the fluoxetine or methylcellulose exposed offspring.

The body mass of the female offspring (F2) from methylcellulose or fluoxetine exposed rats from maternally separated mothers was lowered on PND7 compared to the methylcellulose exposed pups from control mothers. Furthermore, on PND14-28 the methylcellulose exposed pups from maternally separated rats had lower body mass than the other treatment groups. A well known side effect of fluoxetine is weight loss, we did not observe this effect in the female offspring. This might be due to the fewer pups of the fluoxetine-treated mothers, these pups had more access to mother milk. Previous research (Olivier et al., 2011) also showed that body mass was increased as a results of prenatal fluoxetine exposure (PND14, 21, 28, and 35). However this was not the case on PND7, where the fluoxetine exposed pups had a higher body mass. We did observe lower body mass of the female pups from maternally separated dams. This effect disappears from PND 35, suggesting a role for maternal aspects. This is in accordance with the reduced fetal and infant growth found as a result from antenatal depression (El Marroun et al., 2012; Henrichs et al., 2010; reviewed by Stewart 2007).

In male offspring (F2) different effects were found. On PND7 pups from maternally separated dams showed lower body mass than from control mothers if they were exposed to methylcellulose. Moreover, the body mass of fluoxetine exposed pups from control mothers was lower than that of all the other groups. On PND14 and 21 the fluoxetine exposed pups from maternally separated mothers had higher body mass than all other groups, while on PND35, 49, and 56 the methylcellulose exposed offspring from maternally separated mothers had lower body mass than the other groups. Also, a time x maternal treatment x drug interaction was found. It appears that in pups from maternally separated mothers, prenatal fluoxetine exposure led to a higher body mass compared to methylcellulose exposed on PND14, 21, 28, 35, 49 and 56. This effect was not visible in pups

from control mothers. As in the case of the female offspring maternal separation led to a lower body mass, however, this effect started at PND28 and vanished after PND56. The increased body mass for fluoxetine treated rats from maternally separated mothers but not fluoxetine treated rats from control mothers compared to the methylcellulose treated rats could be due to the fact that for the control mothers fluoxetine treatment did not lead to significantly smaller litter sizes, probably due to the low sample size (Supplemental 7). Furthermore the decreased body mass of the methylcellulose exposed pups from maternally separated rats is, as for the females, in accordance with low infant growth found as an effect of human antenatal depression. Though, this effect appears to persist longer than in the female offspring.

Due to the low survival rate of the offspring some treatment groups were not represented at all, and some treatment groups contained only a small number of individuals for the assessment of social play at juvenile age and social interaction during adulthood.

In the social play test no effect was found for the male $SERT^{+/+}$ offspring. In the male $SERT^{+/-}$ offspring, the duration of social exploration was higher as a result from maternal separation of the mother in methylcellulose exposed pups. Moreover, the duration of social exploration was reversed by fluoxetine in the offspring from maternally separated mothers. For the $SERT^{+/+}$ female offspring, methylcellulose exposed offspring had higher frequencies of social exploration, boxing and following when their mothers were maternally separated. This effect was also reversed by fluoxetine in offspring from maternally separated mothers. However, the duration of pouncing was increased as a result from fluoxetine exposure in offspring from maternally separated rats. In the $SERT^{+/-}$ female offspring, fluoxetine exposure led to an increase in time spent on pinning behavior in offspring from maternally separated mothers. It is difficult to draw any firm conclusions for the social play test, because of the small group sizes. Although the results are conflicting to a certain extent, a comprehensive look at the results indicates that maternal separation increases social behavior, which is reversed by fluoxetine treatment. Total duration spent on pouncing in the $SERT^{+/+}$ females and total duration spent on pinning in the $SERT^{+/-}$ females contradicts this theory. However, these typical play behaviors are better defined by frequency than duration (Van Kerkhof et al., 2013). Hence, during scoring of the behaviors, more emphasis was put on determining their frequencies instead of duration, which may have led to inattentive scoring with regard to the duration of these specific behaviors.

In the social interaction test only the behavior of the male $SERT^{+/+}$ offspring was scored, and only methylcellulose offspring from maternally separated and control mothers was evaluated. Maternal separation led to an increase in duration and frequency of pouncing, an increase in the duration of following, and reduction in time spent on passive contact. Thus, even though the data on passive contacts contradicts this, a global look at the results again suggests that maternal separation leads to an increase in social behavior.

Although with cautious interpretation, our results on social play and social interaction suggest that maternal separation has an intergenerational effect, and increases social behavior. Moreover, this effect appears to be reversed by fluoxetine. Little is known about the intergenerational effect of the antenatal depression model (maternally separated $SERT^{+/-}$ mother) on social behavior. Epigenetics are often suggested as a possible mechanism for the intergenerational effect. Low maternal care has shown to lead to altered DNA methylation profiles and increased anxiety-like behaviors (Weaver et al., 2004). Furthermore, recent research (De Palma et al., 2015) suggests a role for intestinal bacteria in the effect of maternal separation. The authors show that maternal separation alters the colonic milieu which leads to intestinal dysbiosis, which then triggers, likely trough the production of microbial metabolites, the abnormal behavioral patterns caused by maternal separation. Since, the microflora of the mother is transferred to the offspring during delivery, this is a possible mechanism for the intergenerational effect.

In the offspring from maternally separated mothers, fluoxetine exposure decreased social behavior in the social play test. SSRIs are well described in literature in reducing social play behavior when prenatally (Olivier et al., 2011) or postnatally (Homberg et al., 2007) administered. However, these studies were in offspring from healthy mothers, and a comparable treatment group could not be included in our assessment of offspring social behavior. The underlying molecular mechanisms remain to be established, but the 5-HT_{1A} receptor plays a potential key role. This receptor is important during early brain development, and involved in neuronal growth and survival (Sikich et al., 1990, Fricker et al., 2005). One of the proposed mechanisms of the therapeutic action of SSRIs is the desentizitation of the 5-HT_{1A} receptors (Pineyro and Blier, 1999). But surprisingly, prenatal and postnatal SSRI exposure are indicated to increase the 5-HT_{1A} receptor sensitivity (reviewed in Olivier et al., 2013). So far, only one study has looked into the possible role for epigenetics in this mechanism. Toffoli et al. (2014) looked into the epigenetic effects of prenatal fluoxetine exposure in rats, and showed that DNA methylation patterns of global DNA methylation were altered in the hippocampus and cortex. However, they only measured after the weaning period and it is not known if these effects are persistent on the long term.

Aggressive behavior was not fully assessed yet, and thus far we cannot evaluate the effect of fluoxetine, we can merely speculate. Postnatal antidepressant treatment is known to impair aggressive behavior (Manhães de Castro et al., 2001). 5-HT_{1A} receptor agonists are known to suppress aggressive behavior (Reviewed in Olivier & Van Oorschot, 2005). As mentioned, there are indications that the 5-HT_{1A} receptor sensitivity is increased due to pre and/or postnatal SSRI exposure. Increased 5-HT_{1A} activation due to the enhanced sensitivity might explain why SSRI exposure leads to attenuated aggression responses.

The strength of this study was that we studied the effects of antenatal depression itself, fluoxetine itself and especially their combination on social behavior. Which is of more

translational value to the human situation than previous studies, since healthy pregnant women do not take antidepressants. A potential limitation of this study is that we started fluoxetine treatment during pregnancy. SSRIs are known to have a delayed onset of action (Taylor et al., 2006), with a continuous improvement at a decreasing rate for at least 6 weeks. This might not be representative for women who continue the intake of SSRIs during pregnancy rather than starting with it during pregnancy. However, if we started fluoxetine treatment before pregnancy it would not be possible to ensure equally long treatment periods, since it is impossible to exactly determine the onset of pregnancy. Furthermore, we did not determine if the females treated with maternal separation actually showed depressive-like behaviors, although this was established in earlier research (Olivier et al., unpublished data). Also, the fluoxetine significantly lowered body mass of the dams, led to lower nest sizes, and altered the growth of the offspring. These are all confounding factors of which the influence on social behavior cannot be excluded. Finally, a major limitation of this study was that we missed the group of fluoxetine exposed offspring from control mothers. Besides, other treatment groups contained only a small number of animals. At this time the data is supplemented, and this should provide us with more inside on the effects of antenatal depression, fluoxetine treatment, and their combination on social behavior of mothers and their offspring.

In conclusion, our study showed that fluoxetine treatment increases the mortality rate of the offspring. Moreover, we showed that our antenatal depression model increased social behavior in the offspring. Pre- and postnatal fluoxetine exposure reversed this effect. Yet, supplementation of our data needs to elucidate this.

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Supplementary data

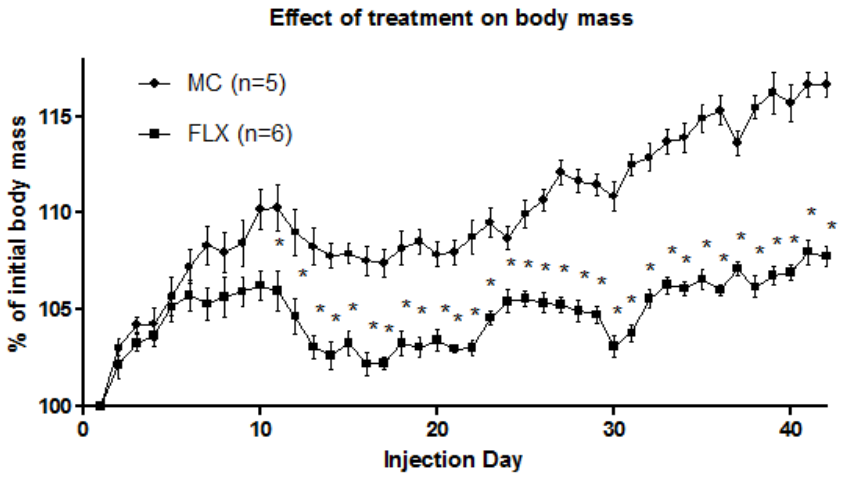
The p-values for differences in body mass of mothers (F1) between different treatment groups.

Treatment day	One way ANOVA F=	P=	C-MC vs C-FLX	C-MC vs MS-MC	C-MC vs MS-FLX	C-FLX vs MS-MC	C-FLX vs MS-FLX	MS-MC vs MS-FLX
1	-	-	-	-	-	-	-	-
2	2.28	n.s.	-	-	-	-	-	-
3	4.53	<0.05	p<0.05	n.s.	p<0.05	P=0.06	n.s.	p<0.01
4	5.80	<0.01	P=0.06	n.s.	p<0.05	p<0.05	n.s.	p<0.01
5	7.33	<0.01	p<0.05	n.s.	p<0.05	p<0.05	n.s.	p<0.01
6	11.65	<0.001	p<0.01	n.s.	p<0.05	p<0.001	n.s.	p<0.01
7	11.12	<0.001	p<0.01	n.s.	p<0.05	p<0.001	n.s.	p<0.01
8	12.54	<0.001	p<0.01	n.s.	p<0.01	p<0.01	n.s.	p<0.001
9	8.159	<0.01	p<0.01	n.s.	p<0.01	p<0.01	n.s.	p<0.01
10	12.41	<0.001	p<0.01	n.s.	p<0.01	p<0.01	n.s.	p<0.001
11	13.13	<0.001	p<0.01	n.s.	p<0.01	p<0.01	n.s.	p<0.001
12	13.52	<0.001	p<0.01	n.s.	p<0.01	p<0.01	n.s.	p<0.001
13	10.71	0.001	p<0.01	n.s.	p<0.01	p<0.01	n.s.	p<0.001
14	12.91	<0.001	p<0.01	n.s.	p<0.01	p<0.01	n.s.	p<0.001
15	9.80	0.001	p<0.01	n.s.	p<0.01	p<0.01	n.s.	p<0.01
16	9.14	0.001	p<0.01	n.s.	p<0.01	p<0.01	n.s.	p<0.01
17	6.82	<0.01	p<0.01	n.s.	p<0.01	p<0.01	n.s.	p<0.01
18	6.43	<0.01	p<0.01	n.s.	p<0.01	p<0.01	n.s.	p<0.01
19	4.85	<0.05	p<0.05	n.s.	p<0.001	p<0.05	n.s.	p<0.05
20	4.76	<0.05	p<0.05	n.s.	p<0.001	p<0.05	n.s.	p<0.05
21	4.78	<0.05	p<0.01	n.s.	p<0.01	p<0.05	n.s.	P=0.06
22	5.60	<0.05	n.s.	n.s.	p<0.01	n.s.	n.s.	p<0.01
23	43.86	<0.001	p<0.001	n.s.	p<0.001	p<0.001	n.s.	p<0.001
24	27.28	<0.001	p<0.001	n.s.	p<0.001	p<0.001	n.s.	p<0.001
25	19.28	<0.001	p<0.01	n.s.	p<0.01	p<0.001	n.s.	p<0.001
26	18.22	<0.001	p<0.01	n.s.	p<0.01	p<0.001	n.s.	p<0.001
27	10.30	0.001	p<0.05	n.s.	p<0.05	p<0.01	n.s.	p<0.001
28	13.38	<0.001	p<0.01	n.s.	p<0.01	p<0.01	n.s.	p<0.001
29	11.30	<0.001	p<0.05	n.s.	p<0.05	p<0.01	n.s.	p<0.001
30	8.96	0.001	p<0.05	n.s.	p<0.05	p<0.01	n.s.	p<0.01
31	6.54	<0.01	p<0.05	n.s.	p<0.05	p<0.05	n.s.	p<0.01
32	8.071	<0.01	p<0.05	n.s.	p<0.05	p<0.05	n.s.	p<0.01
33	4.81	<0.05	n.s.	n.s.	n.s.	P=0.06	n.s.	p<0.01
34	4.42	<0.05	n.s.	n.s.	n.s.	P=0.06	n.s.	p<0.01
35	4.48	<0.05	p<0.05	n.s.	p<0.05	P=0.07	n.s.	p<0.05
36	4.37	<0.05	P=0.07	n.s.	n.s.	n.s.	n.s.	p<0.01
37	5.76	<0.01	p<0.05	n.s.	p<0.05	p<0.05	n.s.	p<0.01
38	6.00	<0.01	p<0.05	n.s.	p<0.05	p<0.05	n.s.	p<0.01
39	4.17	<0.05	n.s.	n.s.	n.s.	n.s.	n.s.	p<0.01
40	5.59	<0.05	p<0.05	n.s.	p<0.05	P=0.06	n.s.	p<0.01
41	5.16	<0.05	P=0.07	n.s.	P=0.07	n.s.	n.s.	p<0.01
42	7.48	<0.01	p<0.05	n.s.	p<0.05	p<0.05	n.s.	p<0.01

S1 *.The p-values for differences in body mass of mothers (F1) between different treatment groups. P values were obtained via a one-way ANOVA for each of the measurement days, with subsequently LSD post hoc testing.*

C: Control handled; MS: Maternally separated; MC: methylcellulose treated; FLX: fluoxetine treated.

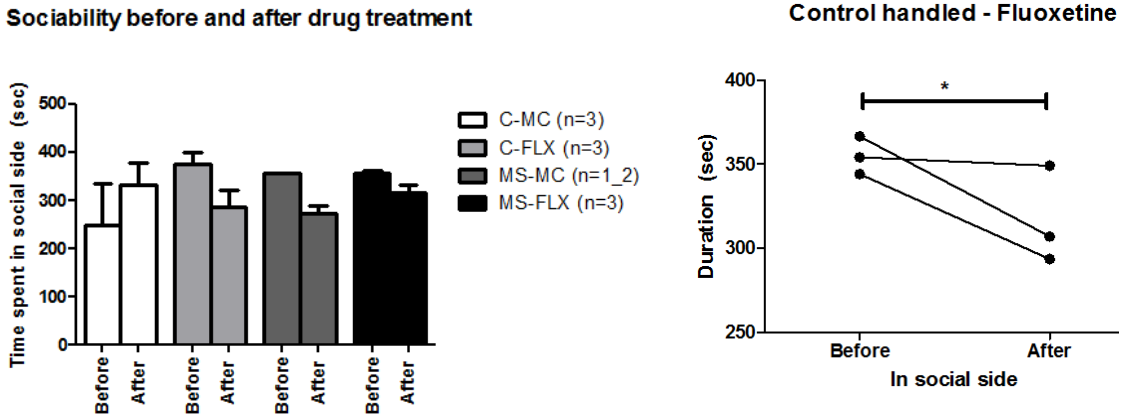
The effect of fluoxetine treatment on body mass – F1 not pregnant



S2 *The effect of fluoxetine on body mass of non-pregnant females expressed as the percentage of the body mass on the starting day of the treatment. Data are presented as mean ±S.E.M. body mass.*

MC: methylcellulose treated; FLX: fluoxetine treated.

The effect of drug treatment on sociability – F1 not pregnant

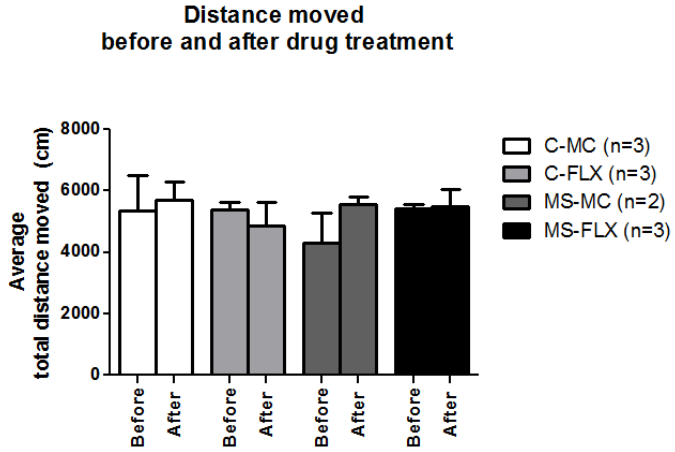


S3 *Left) The effect of fluoxetine in maternally separated and control handled females. The recording of one of the two maternally separated rats prior to the methylcellulose treatment was disturbed. Therefore this group(MS-MC) only contains one female with repeated test data, thus performing a paired t-test in this group was not possible. Data are presented as mean ±S.E.M. time spent in the social side (sec) before and after drug treatment.*

Right) Control handled females spent less time in the social side of the three-chamber apparatus after treatment with fluoxetine ($t_{(1,2)}$= 4.53; p<0.05). Data are presented as duration spent in the social side for each individual before and after the drug treatment.

C: Control handled; MS: Maternally separated; MC: methylcellulose treated; FLX: fluoxetine treated.

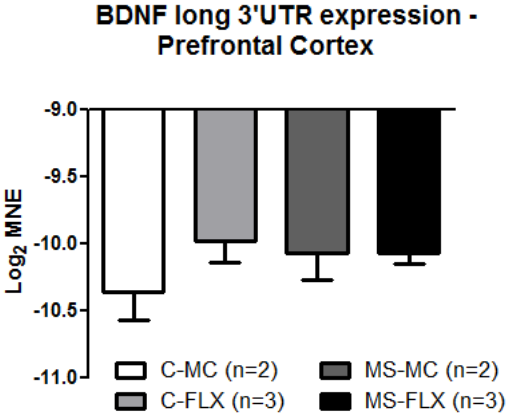
The effect of fluoxetine on maternally separated and control handled rats on total distance moved – F1 not pregnant



S4 *The effect of fluoxetine treatment in both maternally separated and control females on distance moved in a sociability test. Data are presented as mean ±S.E.M. total distance moved before and after the drug treatment.*

C: Control handled; MS: Maternally separated; MC: methylcellulose treated; FLX: fluoxetine treated

BDNF long 3'UTR expression in females – F1 not pregnant



Treatment group Not pregnant	Log2 fold change
C-FLX	0.38
MS-MC	0.29
MS-FLX	0.29

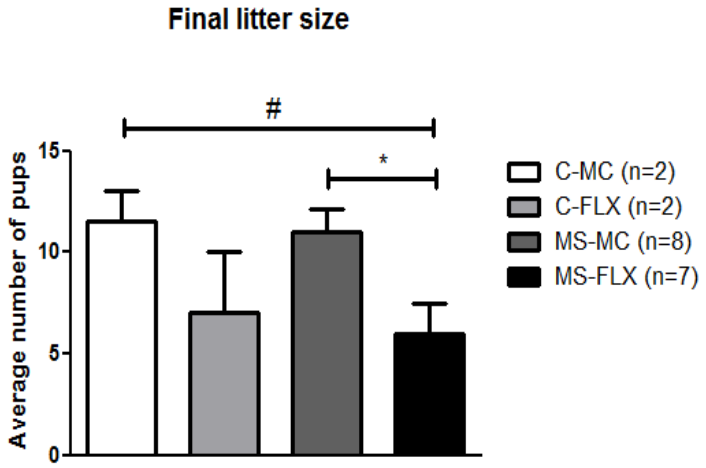
S5 *The effect of maternal separation and/or fluoxetine on BDNF long 3'UTR gene expression in the prefrontal cortex . Data are presented as mean ±S.E.M. log_2 mean normalized expression (MNE).*

C: control handled; MS: maternally separated; MC: methylcellulose treated; FLX: fluoxetine treated.

S6 *Log2 fold change of different treatment groups compared to the control handled and methylcellulose treated group. A negative value indicates a reduction in expression levels.*

C: control handled; MS: maternally separated; MC: methylcellulose treated; FLX: fluoxetine treated.

The effect of fluoxetine treatment on the final litter size of maternally separated and control treated females



S7 *Effect of fluoxetine exposure during pregnancy on the final litter size (F2). A one-way ANOVA revealed a trend ($F_{(1,18)}$=3.11; p=0.06). LSD post hoc testing was used for further analysis. Data are presented as mean ±S.E.M number of pups per litter.*

**p<0.05, #p=0.07*

C: Control handled; MS: Maternally separated; MC: methylcellulose treated; FLX: fluoxetine treated.

P values for differences in body mass of the offspring- F2

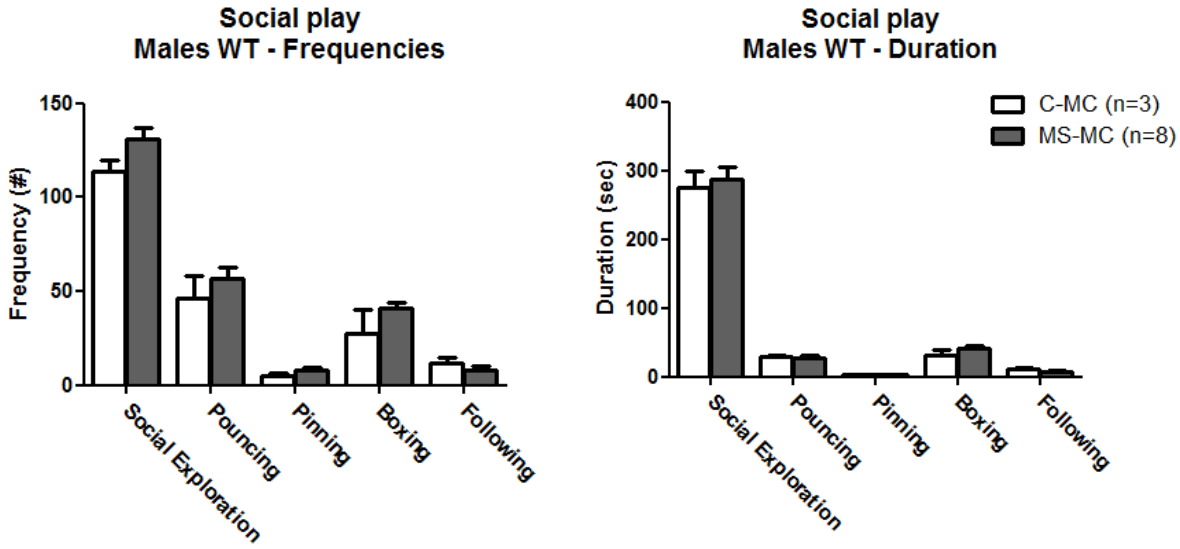
Females	PND7	PND14	PND21	PND28
C-MC vs C-FLX	n.s.	n.s.	n.s.	n.s.
C-MC vs MS-MC	0.014	0.048	0.076	0.016
C-MC vs MS-FLX	0.030	n.s.	n.s.	n.s.
C-FLX vs MS-MC	0.074	0.004	0.006	0.000
C-FLX vs MS-FLX	n.s.	n.s.	n.s.	n.s.
MS-MC vs MS-FLX	n.s.	0.001	0.005	0.000

Males	PND7	PND14	PND21	PND28	PND35	PND49	PND56
C-MC vs C-FLX	0.001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
C-MC vs MS-MC	0.009	n.s.	0.059	0.000	0.01	0.000	0.020
C-MC vs MS-FLX	n.s.	0.004	0.031	n.s.	n.s.	n.s.	n.s.
C-FLX vs MS-MC	0.066	n.s.	n.s.	0.000	0.024	0.019	n.s.
C-FLX vs MS-FLX	0.01	0.002	0.042	n.s.	n.s.	n.s.	n.s.
MS-MC vs MS-FLX	n.s.	0.000	0.000	0.000	0.001	0.003	0.011

S8 *The p-values for differences in body mass of female (above) and male (below) offspring (F2) between different treatment groups. Only days that had at least one significant difference between two groups are shown. P values were obtained via a one-way ANOVA for each of the measurement days, with subsequently LSD post hoc testing.*

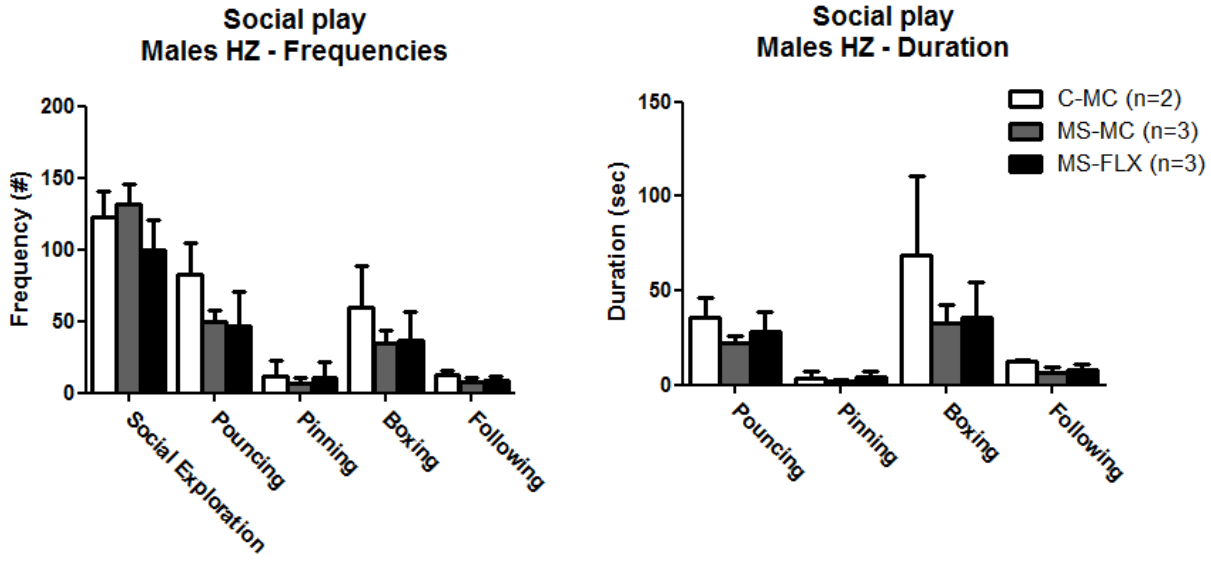
C: control handled; MS: maternally separated; MC: methylcellulose treated; FLX: fluoxetine treated.

The (non-significant) effects of maternal separation of the mother and/or fluoxetine exposure on social play in wildtype and heterozygous male offspring – F2



S9 *The (non-significant) effects of maternal separation of the mother and/or fluoxetine exposure on the different behaviors of wildtype (WT) male offspring in the social play test. Frequency's of and duration spent on a certain behavior are shown on the left and right respectively. Data are represented as mean ±S.E.M.*

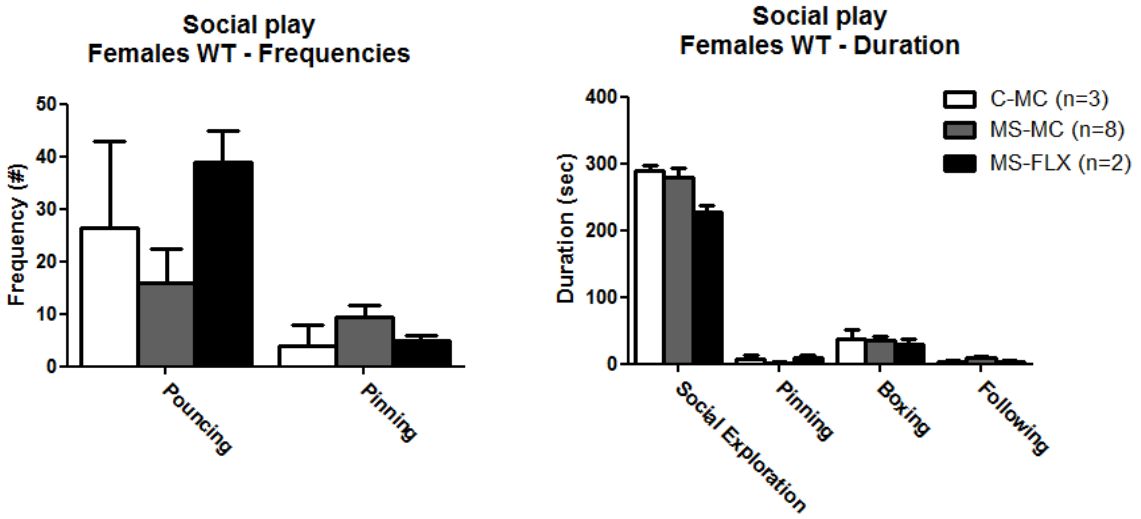
C: Control handled: MS: Maternally separated: MC: methylcellulose treated: FLX: fluoxetine treated.



S10 *The (non-significant) effects of maternal separation of the mother and/or fluoxetine exposure on the different behaviors of heterozygous (HZ) male offspring in the social play test. Frequency's of and duration spent on a certain behavior are shown on the left and right respectively. Data are represented as mean ±S.E.M.*

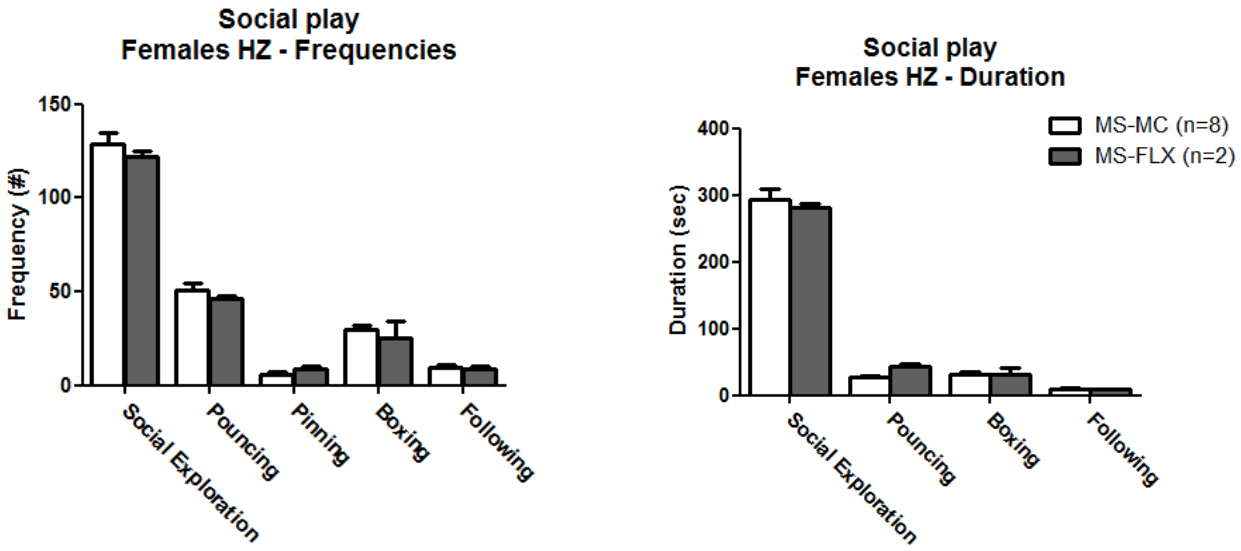
C: Control handled: MS: Maternally separated: MC: methylcellulose treated: FLX: fluoxetine treated.

The (non-significant) effects of maternal separation of the mother and/or fluoxetine exposure on social play in wildtype and heterozygous female offspring – F2



S11 *The (non-significant) effects of maternal separation of the mother and/or fluoxetine exposure on the different behaviors of wildtype (WT) female offspring in the social play test. Frequency's of and duration spent on a certain behavior are shown on the left and right respectively. Data are represented as mean ±S.E.M.*

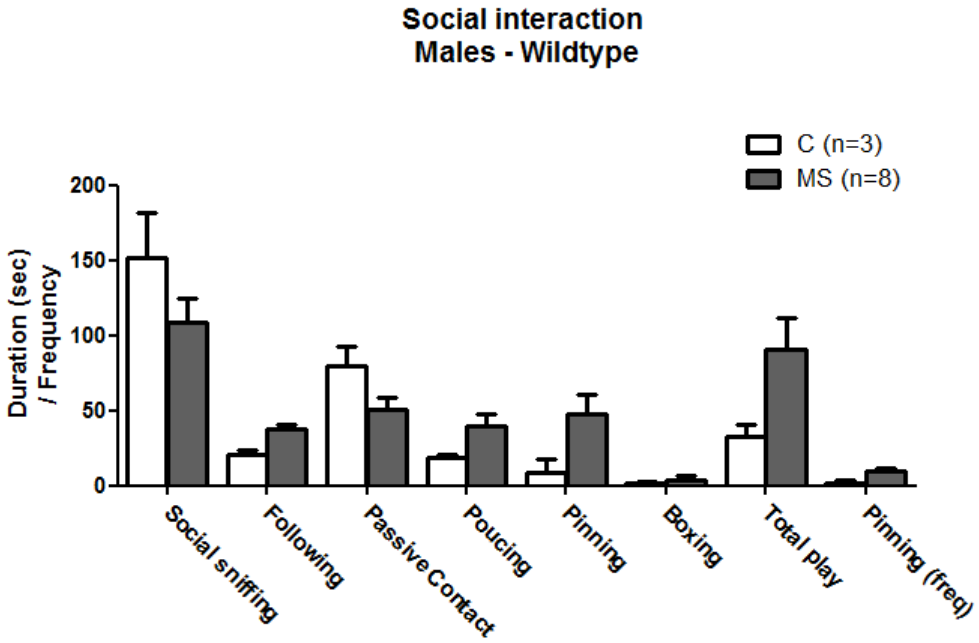
C: Control handled; MS: Maternally separated; MC: methylcellulose treated; FLX: fluoxetine treated.



S12 *The (non-significant) effects of maternal separation of the mother and/or fluoxetine exposure on the different behaviors of heterozygous (HZ) female offspring in the social play test. Frequency's of and duration spent on a certain behavior are shown on the left and right respectively. Data are represented as mean ±S.E.M.*

C: Control handled; MS: Maternally separated; MC: methylcellulose treated; FLX: fluoxetine treated.

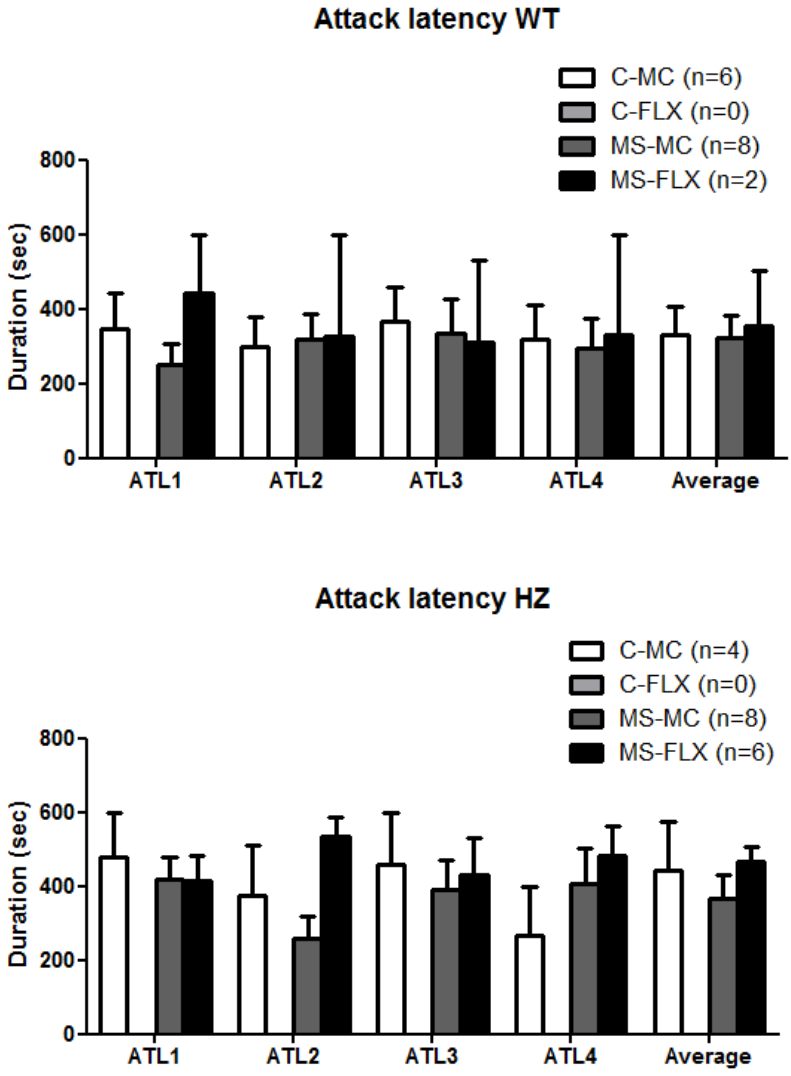
The (non-significant) effects of maternal separation on social interaction in the offspring – F2 wildtype males, methylcellulose treated



S13 *The (non-significant) effect of maternal separation of the mother on social interaction of the male wildtype offspring exposed to methylcellulose. Data are presented as mean ±S.E.M. time spent on or frequency of (pinning) a certain behavior.*

C: Control handled; MS: Maternally separated

Attack latency's of the males in the resident intruder test –F2



S14 *.No effect of maternal separation of the mother and/or fluoxetine exposure on attack latency was found. Above the attack latency of the wildtype males is shown, and below the attack latency of the heterozygous males. Since the test only took 10 minutes, the maximum attack latency was 600 sec. Data are represented as mean ±S.E.M. attack latency.*

C: Control handled; MS: Maternally separated; MC: methylcellulose treated; FLX: fluoxetine treated.