

Perinatal fluoxetine exposure changes social and stress-coping behavior in adult rats housed in a seminatural environment

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Abstract

The use of selective serotonin reuptake inhibitors (SSRI) during pregnancy has increased tremendously, but the consequences for the offspring remain largely unclear. Several studies have described potential effects of perinatal SSRI-exposure on neurobehavioral outcomes using simplified rodent test set-ups, however these set-ups only assess a small fraction of the behavior. For translational purposes it is important to take the environmental influences into account which children are exposed to in real life. By using a seminatural environmental set-up, this study is the first to assess behavioral outcomes in offspring exposed to perinatal SSRI exposure under seminatural circumstances. Mothers received daily the SSRI fluoxetine (FLX, 10 mg/kg p.o.) or vehicle (CTR) from gestational day 1 until postnatal day 21. To assess the effect of FLX exposure during early development, female and male offspring were behaviorally tested in the seminatural environment at adulthood. Baseline behavior was measured in addition to responses during and after stressful white-noise events. Behavior was observed on two days, day 4 on which females were sexually non-receptive, and day 7, on which females were sexual receptive. Perinatal FLX exposure reduced general activity in females and increased behavior related to a social context in both males and females. After a stressful white-noise event some behaviors switched. Whereas FLX-females switch from resting socially to resting more solitarily, FLX-males show an increase in self-grooming behavior after the stressor and showed more freezing behavior in the open area. We conclude that perinatal FLX exposure leads to alterations in social and stress-coping behaviors in adulthood, when observed in a seminatural environment. Whether these adaptations in behavior are advantageous or disadvantageous remains to be established.

1. Introduction

Depressive symptoms frequently occur during pregnancy and can affect the developing child in a profound way. Over the last years, selective serotonin reuptake inhibitors (SSRIs) have gained acceptance as medication during pregnancy, which resulted in an increase in the prescription rate in pregnant women (Alwan et al., 2011; Ververs et al., 2006). However, antidepressants can cross the placenta and are present in breast milk (Kristensen et al., 1999; Rampono et al., 2004). As a result, a growing number of children is being exposed to SSRIs during the perinatal period (Kim et al., 2006; Noorlander et al., 2008).

By blocking the serotonin transporter (SERT), SSRIs inhibit the reuptake of serotonin (5-HT) into the presynaptic nerve terminals, which results in an increase in the synaptic concentration of 5-HT. During adulthood, 5-HT mainly acts as a modulatory neurotransmitter regulating emotion, stress responses, sleep, learning, cognition, and attention (Canli and Lesch, 2007). During early brain development, on the other hand, 5-HT also acts as a neurotrophic factor, regulating cell division, differentiation, migration, and synaptogenesis (Azmitia, 2001; Gaspar et al., 2003). Therefore, it is assumed that changes in 5-HT levels during *in utero* neurodevelopment have the potential to affect these processes as well as subsequent serotonergic function and vulnerability to affective disorders (Lesch and Mossner, 1998).

Several studies in humans have described an effect of antenatal SSRI-exposure on neurobehavioral outcomes. For example, SSRI treatment during pregnancy has been associated with disturbed sleep patterns, affected social-emotional development, and increased internalizing and externalizing behavior in the offspring (Brandlistuen et al., 2015; Oberlander et al., 2010; Weikum et al., 2013). Recently, an increased risk for autism spectrum disorder (ASD) in offspring was added to this list (Boukhris et al., 2016; Rai et al., 2013). ASD can be characterized by e.g. difficulties in social interaction and communication and a tendency to engage in repetitive

behaviors. The problem with these human studies, though, is the difficulty to discern between the effects of the SSRIs and the effects of the mothers' underlying depression. In fact, when controlled for maternal mood and stress, this link between antenatal SSRI use and the occurrence of ASD in the offspring does not prevail (Brown et al., 2017). Still, it is difficult in human studies to control for all potential environmental influences. Animal models, on the other hand, can be used to study the effects of SSRI use on the neurodevelopmental outcomes in the offspring without interference of potential confounders.

Several studies have shown that SSRI exposure during development can alter social behavior: in juvenile rats, social play behavior with an unfamiliar play partner is reduced after perinatal SSRI exposure, (Houwing et al., 2019b; Khatri et al., 2014; Olivier et al., 2011b; Rodriguez-Porcel et al., 2011; Simpson et al., 2011). Furthermore, SSRI exposure throughout pregnancy and lactation can increase aggressive behavior in adult male mice (Kiryanova and Dyck, 2014; Svirsky et al., 2016), while postnatal SSRI exposure has the potential to reduce sexual behaviors in rodents (Gouvea et al., 2008; Harris et al., 2012; Rayen et al., 2013; Rodriguez-Porcel et al., 2011). Unfortunately, there are still a lot of discrepancies between the different studies, some of which can be explained by the timing of the SSRI exposure. In the adolescent and adult brain the SERT is only expressed in neurons of the raphe nucleus, but at early developmental stages the SERT expression pattern is more widespread (Homberg et al., 2010; Olivier et al., 2011a). Altered activation of these transporters, and thus the serotonergic tone, could lead to changes in brain development. Besides, although the transient SERT expression disappears during the early postnatal phase, 5-HT retains its neurotrophic actions. It has been suggested that inhibition of SERT and excess 5-HT exposure during a critical period in fetal development leads to alterations in monoamine systems throughout various brain regions and results in long lasting neurological effects which differ throughout lifespan (Homberg et al.,

2010; Weinstock, 2015). While some studies exposed the offspring to SSRIs prenatally, others used a postnatal approach. The different timing of the SSRI exposure could, in theory, induce different behavioral outcomes due to the different patterns of SERT expression (Ansorge et al., 2004; Popa et al., 2008).

In our current experiment, we circumvented the different potential outcomes of SSRI exposure on critical time points, by administering pregnant females daily with fluoxetine (SSRI) or vehicle from gestational day 1 (GD1) until the pups are weaned at postnatal day (PND) 21. This timeframe was chosen to resemble the entire human pregnancy period and part of the postnatal period, since rat brain neurodevelopment at postnatal days 1–10 equals the third trimester of pregnancy in humans (Andrews and Fitzgerald, 1997; Dobbing and Sands, 1979). Thus, we were able to investigate the neurodevelopmental effect of SSRI treatment during pregnancy on the offspring in a way that is translational to the human situation.

To bypass another limitation of previous studies, our experiment used a seminatural environmental set-up in which rats live in groups for several days and can express all aspects of their natural behavior (Bove et al., 2018; Buwalda et al., 2017; Le Moëne and Ågmo, 2018, 2019). This way, the behavioral alterations in the offspring due to perinatal SSRI exposure can be investigated in a social context, in which the consequences of environmental influences and life-events can be determined. Simplified rodent test set-ups can only investigate a small fraction of the behavior and fail to take into account the environmental influences children are exposed to in real life. The social interaction test, for example, investigates the time two paired rats sniff and groom each other, as an indicator of social behavior. However, the rats are paired in a small controlled test arena which does not allow them to escape from the situation. In real life, people can decide to (socially) interact with one or another, or simply withdraw from social interaction. Environmental factors can influence the decisions that are being made at that moment. Therefore,

the seminatural environmental approach used in our study is a more translational test set-up in which the full repertoire of behavior can be expressed and investigated.

Our test approach allows to study the same group of rats (cohort) over time in a seminatural setting without a change in test environment (e.g. the transport from homecage to test cage is stressful by itself). In order to investigate the consequences of experiencing stressful life-events, we simulated such an event in the seminatural environment by exposing the rats to a 10-minute lasting 90 dB white-noise episode. White-noise is comparable to the sounds of the natural predator of rats, rattlesnakes, which induces physiological and behavioral responses associated with stress (Rowe et al., 1986; Weyers et al., 1994). By doing so, we were able to investigate the behavioral adaptation caused by perinatal SSRI exposure on baseline levels in combination with studying the behavioral changes during and after a stressful event. In addition, because the hormonal status of females can have an effect on their behavior, we controlled for the estrous cycle of females. We observed the behavior of both males and females before, during and after the stressor on a day with females in diestrus and on a day when in proestrus (induced with hormonal treatment). We hypothesized that perinatal SSRI exposure would reduce components of social behavior in the offspring based on results found in simplified rodent tests (Khatri et al., 2014; Olivier et al., 2011b; Rodriguez-Porcel et al., 2011; Simpson et al., 2011; Zimmerberg and Germeyan, 2015). During and after the stressor, we expected that FLX exposed animals would display increased freezing behavior based on the increased anxiety-levels found in rats in classic tests assessing anxiety-like behaviors (Olivier et al., 2011b). As sex differences are prominent after early-life events, we assess both males and females and expect to find differences in the responses to the perinatal SSRI treatment, where responses in males are more robust than in females based on results found in simplified rodents tests (Houwing et al., 2019b).

2. Material and Methods

2.1 Animals and dam housing conditions

A total of ten female and ten male Wistar rats (weighing 200-250 g at the time of arrival) were obtained from Charles River (Sulzfeld, Germany) for breeding. They were used as dams and potential father of the offspring. These animals (but also the future offspring) were housed in same sex pairs in Makrolon® IV cages in a room with controlled temperature (21 ± 1 °C) and humidity (55 ± 10 %) on a 12:12 h light/dark cycle (lights on 11:00 h). Commercial rat pellets (Standard chow from SDS, Special Diet Services) and tap water were provided ad libitum, and nesting material was presented.

All experimentation was carried out in agreement with the European Union council directive 2010/63/EU. The protocol was approved by the National Animal Research Authority in Norway.

2.2 Breeding and antidepressant treatment

Prior to breeding, females were checked daily for their estrus cycle stage by placing them together with a male rat for maximum 5 minutes. They were considered receptive when they responded to a mount with a lordosis response. When receptive, the females were placed with a male for approximately 24 hours (Gestational day 0). During this period, each female-male couple was housed in a Makrolon® IV cage. After 24 hours, both male and female returned to their original homecage (with same-sex partner) for the first two weeks of pregnancy. On gestational day 14, the females were housed singly in Makrolon® IV cages with access to nesting material.

From gestational day 1 (G1) until postnatal day 21 (PND21), females were administered daily with either 10 mg/kg fluoxetine (apotekproduksjon, Oslo, Norway) or a vehicle

(Methylcellulose 1%, (Sigma, St. Louis, MO, USA)) using gavage with a stainless steel feeding needle (total of 6 weeks). Fluoxetine tablets (for human usage) were pulverized and dissolved in sterile water (2mg/mL) and injected at a volume of 5mL/kg. As control condition, methylcellulose, the non-active filling of a fluoxetine tablets, was dissolved in sterile water to create a 1% solution and administered at a volume of 5mL/kg as well. The amount of vehicle/fluoxetine given was adjusted upon the weight of the females who were weighed every three days. The dose of fluoxetine was based on comparison to human situations (Lundmark et al., 2001; Olivier et al., 2011b). Near the end of pregnancy, dams were checked twice a day (9:00 h and 15:00 h) for pup delivery.

2.3 Offspring housing conditions before the seminatural environment

After birth, litters were not culled. Pups were weaned at PND 21 and housed in groups of two or three same sex littermates in Makrolon IV cages (see Table S2 for more details). Ears were punched for individual recognition. Until introduction into the seminatural environment (at 13-18 weeks of age), offspring were left alone and only handled during weekly cage cleaning. Only female rats were “disturbed” for the ovariectomy surgery two weeks before introduction to the environment (see 2.4).

2.4 Ovariectomy surgery

Female offspring were ovariectomized to be able to control their estrous cycle with hormone injections. This allowed us to 1) control for the hormonal state (diestrus versus proestrus) when exploring the effects of perinatal SSRI exposure in females, and 2) to induce sexual receptivity on day 7 to study the effects on sexual behavior, and 3) to limit interference of copulation (a behavior often dominant to other behaviors) on the other days.

Females were given isoflurane anesthesia and were placed on their ventral surface. In addition, buprenorphine (.05 mg/kg) and Carprofen (5mg/kg) were given subcutaneously in the upper neck region of the animal before surgery. Ovariectomy was preceded by a 1-2 cm longitudinal midline dorsal skin incision at the lower back of the animal. Muscle incisions were made bilaterally and the peritoneal cavity was accessed. The ovary was located, the connection between the fallopian tube and the uterine horn ligated, and the ovary was extirpated. Muscle incisions were sutured and a wound clip was placed for skin closure. Animals were given Carprofen (5mg/kg subcutaneously) 24 and 48 hours after surgery. Female offspring were singly housed for 3 days during recovery before returning to their homecage.

2.5 Design

For the behavioral observations, five cohorts of eight rats (offspring) were used, with one cohort in the seminatural environment at the time, thus using 5 different cohorts. A cohort of rats consisted of four males and four females, each sex consisting of two rats from control mothers and two from fluoxetine treated mothers. This resulted in ten animals per treatment group coming from 5 batches for data analysis; 10 females and 10 males that were exposed to fluoxetine during development (FLX-females and FLX-males, respectively), and 10 females and 10 males that were exposed to vehicle during development (CTR-females and CTR-males, respectively).

Within a cohort, same sex animals came from different litters. However, within a cohort, almost every animal had 0-1 sibling from the opposite sex ((see TableS1 for more details)), due to a limited amount of litters available. These littermates, however, were housed in different home cages after weaning. Animals were otherwise unfamiliar to each other and sexually naive.

2.6 Procedure

The day before introduction to the seminatural environment (see 2.7 for description of the environment), offspring were shaved and marked under isoflurane anesthesia for individual recognition on video (the wound clips of the females were also removed at the same time). For both sexes, a square area of approximately 4 x 4 cm was shaved either on the upper back/neck, middle back, lower back or the animal was not shaven at all. In addition, the tails of the females were marked with either 1, 2, or 3 rings (0.5 cm) around the base of the tail using a permanent black marker. Female number four received staining at the tip of the tail (approximately 3 cm). The males received the same markings but the rings were broader (about 1 cm) and male four received an extra ring below the marking of the tail tip. In addition, body weight of the animals was measured. No differences in bodyweight were found between CTR-rats and FLX-rats at this moment.

The offspring were placed in the seminatural environment for 8 days (day 0 – day 8) when adult. Since offspring were entering the seminatural environment in cohorts, the age varied between 13 to 18 weeks. An overview of the whole procedure from the beginning of antidepressant treatment until the end of testing of the offspring is given in Figure 2. Each cohort of animals was introduced on the first day (Day 0) at 10:00 h by placing first the females followed by the males in the open field. On day 8, the animals were taken out from the burrow system at 10:00 h, the end of the experiment. After removal, animals were weighed again (again no significant differences between CTR-rats and FLX rats), and underwent whole animal perfusion fixation. Brains were removed and stored for potential further analysis (not included in this study).

Hormone injections were administered to the females on day 5 (estradiol benzoate) and day 7 (progesterone) at 10:00 h (See 2.8 for more details). During the experiment the seminatural

environment was not cleaned, but between colonies, the seminatural environment was thoroughly cleaned to remove olfactory cues from previous animals.

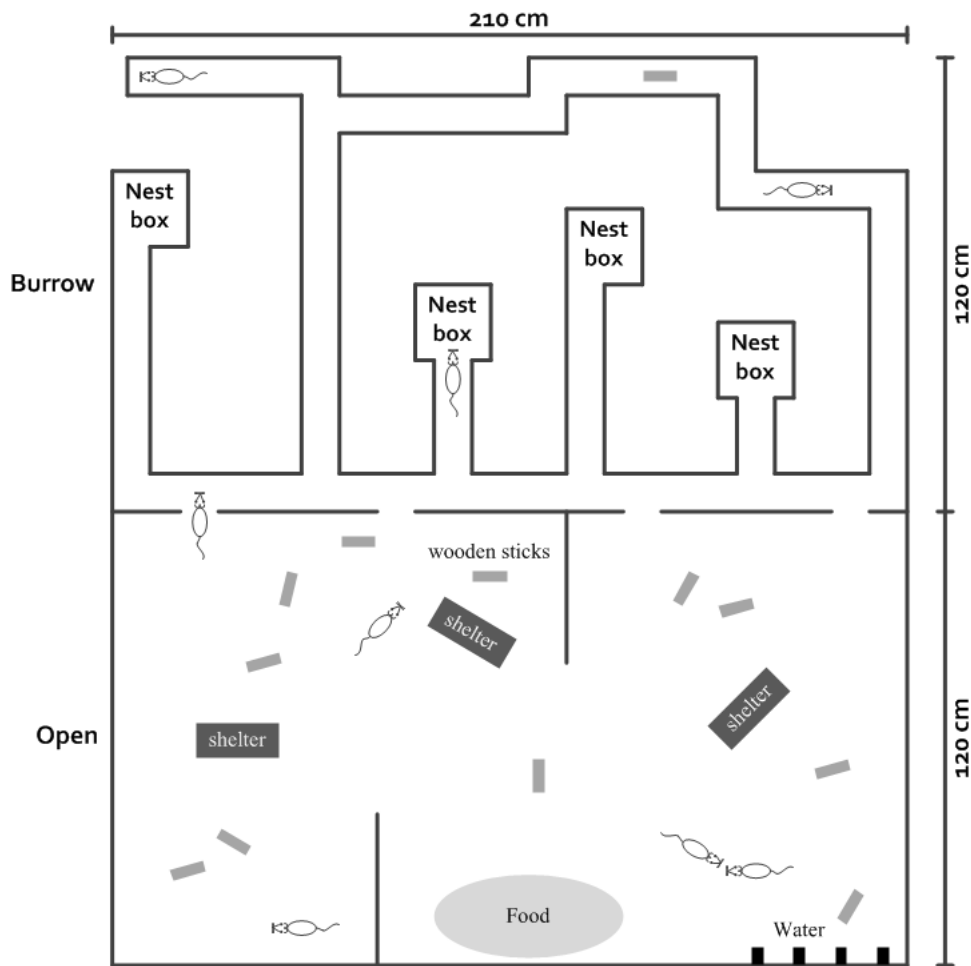
2.7 Seminatural environment

The seminatural environment (2.4 x 2.1 x 0.75 meters) consisted of a burrow system and an open field area which were connected by four 8 x 8 cm openings (Figure 1) (Chu and Agmo, 2014; Snoeren et al., 2015). Several tunnels (7.6 cm wide and 8 cm high) and four nest boxes (20 x 20 x 20 cm) were present in the burrow system. The burrow system was covered with Plexiglas while the 75cm high open area was left open. The open area also had two partitions (40 x 75 cm) to create obstacles simulating the nature. Even though the animals were able to move freely between the open area and burrow system, a curtain between the arenas allowed the light intensity for both arenas to be controlled separately. While the burrow system remained in total darkness for the complete day, a day-night cycle was simulated in the open area. A lamp 2.5 m above the center of the open area provided light (180 lux) from 22:45 h to 10:30 h (simulating day light). From 10:30 h to 11:00 h the light intensity gradually decreased to approximately 1 lux, the equivalent of full moonlight. Similarly, the light gradually increased again from 1 to 180 lux from 22:15 h to 22:45 h.

Both the open area and the burrow system were covered with a 2 cm layer of aspen wood chip bedding (Tapvei, Harjumaa, Estonia) and nest boxes were provided with 6 squares of nesting material each (nonwoven hemp fibers, 5 x 5 cm, 0.5 cm thick, Datesend, Manchester, UK). In the open area 3 red polycarbonate shelters (15 x 16.5 x 8.5 cm, Datesend, Manchester, UK) were placed and 12 aspen wooden sticks (2 x 2 x 10 cm, Tapvei, Harjumaa, Estonia) were randomly distributed. Food was provided in one big pile of approximately 2 kg, in front of the

open area wall opposite of the openings. Water was available *ad libitum* in four water bottles located in the lower right corner of the open field.

Figure 1. Overview of the seminatural environment



Video cameras were mounted on the ceiling 2 m above the seminatural environment: one above the open field (Basler) and an infrared video camera above the burrow system (Basler). Videos were recorded using Media Recorder 2.5. Cameras were connected to a computer and data was (immediately) stored on an external hard drive. Every 24 h, the recording was manually stopped and restarted to create recordings with a length of 24h. This was done to make sure that

when a recording error should occur during the 8 day period, only one recording day would be lost.

2.8 Hormone treatment

During the experiment, female rats were shortly taken out of the seminatural environment on day 5 and 7 in order to receive a subcutaneous hormone injection. The ovariectomized females received 18 µg/kg estradiol benzoate on day 5, and 1 mg of progesterone on day 7. Injections were given at 10:00 h and females were placed back at the same place into the burrow part of the seminatural environment. Since the males did not receive any hormone injections, they were left undisturbed in the seminatural environment. The doses of estradiol and progesterone were based on previous research showing that it produces maximal receptivity and high intensity of female reproductive behavior (see (Spiteri et al., 2010)).

Estradiol benzoate and progesterone (Sigma, St Louis, MO, USA) were dissolved in peanut oil (Apoteksproduksjon, Oslo, Norway) and injected in a volume of 1 ml/kg.

Figure 2. Schematic overview of all experimental procedures

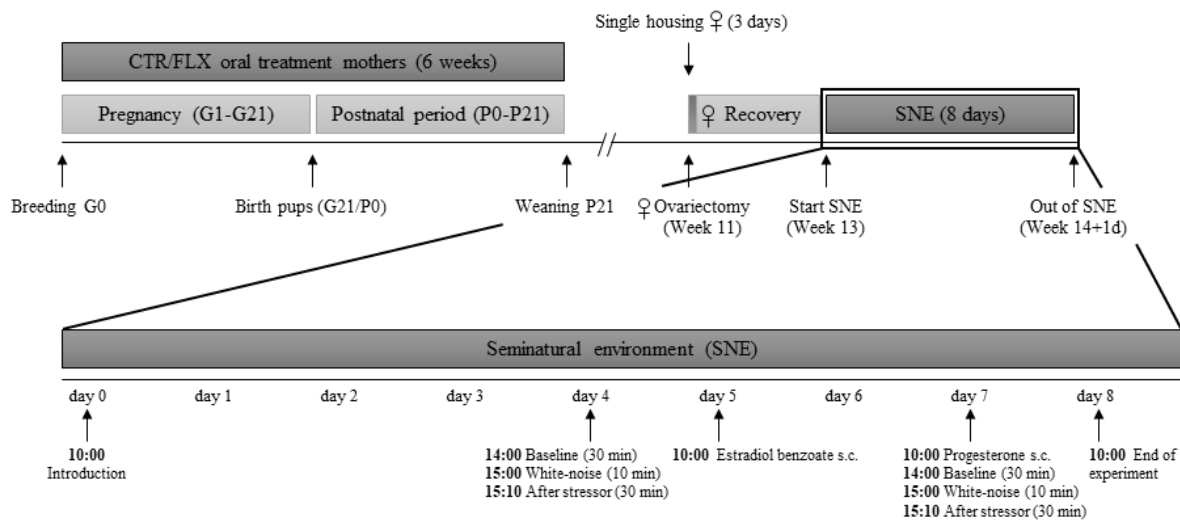


Figure 2: Schematic overview of all experimental procedures. CTR = control, FLX = fluoxetine, G = gestational day, P = postnatal day, SNE = seminatural environment.

2.9 White-noise

To investigate the response of the offspring to a stressful event, they were exposed to loud noise using 90dB white-noise, produced by a white-noise generator (Lafayette instruments, Lafayette, IN) connected to two loudspeakers (Scan-Speak Discovery 10F/8414G10, HiFi Kit Electronic, Stockholm) from which one was placed in the open field and one in the burrow area. Loud noise is often used as stressor in pharmacological and behavioral studies because it produces a strong fear response in rats (Weyers et al., 1994). In addition, white-noise is a similar sound to rattlesnake rattles (Rowe et al., 1986). Since rattlesnakes are predators for rats, this creates immediately a simulation of a natural fear situation. Exposure to white-noise occurred on day 4 (without hormones) and on day 7 (when females were receptive) at 15:00 h and lasted for 10 minutes.

2.10 Behavioral analysis

The frequency and/or duration of a wide variety of behaviors was scored by an observer blind for the treatment of the animals (for the different behaviors, see Table 1). These behaviors were scored on various time points:

- 1). Baseline behavior on day 4 – 30 minutes - the females were without hormones (diestrus)
- 2). Behavior during exposure to white-noise on day 4 – 10 minutes
- 3). Behavior directly after the white-noise on day 4 – 30 minutes
- 4). Baseline behavior on day 7 – 30 minutes - the females were in proestrus and thus sexual receptive
- 5). Behavior during exposure to white-noise on day 7 – 10 minutes
- 6). Behavior directly after the white-noise on day 7 – 30 minutes

Baseline behavior was scored on day 4, after which rats had been habituated to their environment (day 0 – day 3) and exploratory behavior was reduced. Baseline observations on day 4 and 7 started at 14:00 h and lasted for 30 minutes. This specific time point was chosen because females are most receptive 4 hours after the progesterone injection (on day 7) (Glaser et al., 1983). To keep scoring time points the same, we chose the same time point on day 4 as well.

White-noise exposure on day 4 and 7 started at 15:00 h and lasted for 10 minutes. These 10 minutes during white-noise, and the following 30 minutes thereafter were scored separately. The frequency and/or duration of a wide variety of behaviors was scored by an observer blind for the treatment of the animals (Table 1). In addition, the location of the animal was scored: in the open field or in the burrow system. During interactions with other animals, the interacting partner was also noted. All behavioral scoring was done using the Observer XT, version 12 (Noldus, Wageningen, the Netherlands). One 30-minute session was scored by 3 independent observers to calculate the interobserver correlation with a Spearman's rho, which turned out to be 0.93.

Table 1. Ethogram of observed behaviors in the seminatural environment

Behavior	Description
Walking	Walking through the environment
Running	Running with speed through the environment
Walking over/under	Walking over or under another animal
Pursuing	Moving or running forward in the direction of a conspecific
Nonsocial exploration	Exploring the environment by sniffing, usually when slowly walking or sitting still
Resting/immobile alone	Sitting or sleeping with minimal movement of the head without other rats in close vicinity
Resting/immobile socially	Sitting or sleeping with minimal movement of the head with at least 1 other rat on maximum 1 rat body length away
Hiding in shelter alone	Being in the shelter alone
Hiding in shelter socially	Being in the shelter with at least one other rat
Allogrooming	Grooming any part of the partners body, usually on the head or in the neck region
Sniffing anogenitally	Sniffing the anogenital region of the conspecific
Sniffing other rat	Sniffing any part of the conspecifics body, except for the anogenital region
Pouncing	Jumping onto the neck of the partner, usually followed by a nuzzling movement. Usually occurs very short and rapid
Pinning	Usually in response to pouncing, the partner rotates into a supine position, while the other animal is standing over it
Boxing/wrestling	One or both animals are pushing, pawing and grabbing at each other using their forepaws
Nose-off	Facing another rat, usually in a tunnel, resulting in one rat moving forwards and the other backing up
Fighting	Forming a tight ball with another rat, rolling around while biting.
Kicking	Kicking at another rat using the hind paws
Mount	Mounting on the rump of another rat from behind with pelvic thrusting
Intromission	Mounts including penile insertion
Ejaculation	Penile insertion lasts longer than at intromission and is associated with rhythmic abdominal contractions
Paracopulatory behavior	Female approaching a male followed by runaway, often associated with hops, darts, ear wiggling
Lordosis	Receptive behavior with a hollow back and deflect of tail to one side
Carrying nesting material	Playing with or carrying around nesting material
Carrying wood sticks	Playing with or carrying around the wooden stick
Pushing bedding	Moving the bedding material around
Self-grooming	Paw strokes made by the nose and ears, followed by body licking
Postcopulatory self-grooming	Self-grooming immediately after copulation
Eating	Eating, usually while sitting
Drinking	Drinking from one of the bottles in the open field
Freezing	Complete absence of movement in addition to a tense body posture
In opening	Standing in one of the openings connecting the open field and the burrow system and watching to the other side
Rearing	Exploring while raising itself upright on its hind paws
Fleeing	Running away from another rat with high speed

Behavioral clusters of observed behaviors in the seminatural environment	
Activity (non-socially)	Combines walking, walking over/ under, running, pursuing, and nonsocial exploration
Passive behavior	Combines resting alone, resting socially, hiding alone, and hiding
Social context	Combines socially active behavior <i>and</i> socially passive behavior
Socially active behavior	Combines pinning, pouncing, sniffing anogenitally, allogrooming, sniffing other rats
Socially passive behavior	Combines hiding socially, and resting socially
Conflict behavior	Combines nose-off, fighting, kicking, and boxing/wrestling

2.11 Statistical analysis

As indicated in table 1, behavioral clusters were created beforehand by grouping relevant behaviors. These behavioral clusters and the separate behavioral data from the open field, the burrow system and the total environment were analyzed in different ways. First, the behavior observed on day 4 and 7 were combined and analyzed for the periods “baseline”, “white-noise exposure”, and “after stressor”. Then, the behavior on day 4 and 7 were analyzed separately for the same periods.

A Shapiro–Wilk test showed no homogeneity of variance. All behavioral data were therefore analyzed using the nonparametric Mann–Whitney U test to compare FLX-rats with CTR-rats. The Wilcoxon test was used when the different test periods were compared.

Since relatively few litters were used, a Kruskal-Wallis test was performed to check for possible litter effects, which were not found.

3. Results

Since we explored all the behaviors that the rats performed in the seminatural environment, this experiment generated a lot of data. It is, therefore, impossible to discuss all the behaviors separate in this result section. A complete overview of all the behaviors at the different test moments can be found in Table S2 of the supplementary materials.

Most behavioral differences were found in females rats. The difficulty when studying females is that their behavior is largely depending on their estrous cycle phase (hormonal state). In this experiment, we controlled for the hormonal state and tested them during diestrus (day 4) and during proestrus (day 7), and the data was presented separately. However, analysis of the data in which both days are combined, and thus without taking into account the hormonal state of the female, is maybe most similar to the natural situation in which females can be in different phases of their estrous cycle. Therefore, the description of these results can also be found in the supplemental materials (Results SR1).

3.1 Baseline behavior

First, we were interested in the effects of perinatal FLX exposure on the baseline behaviors in male and female rats compared with CTR-rats. Therefore, we analyzed the behavioral data for 30 minutes on day 4 and day 7 at 14:00 h, during the dark phase.

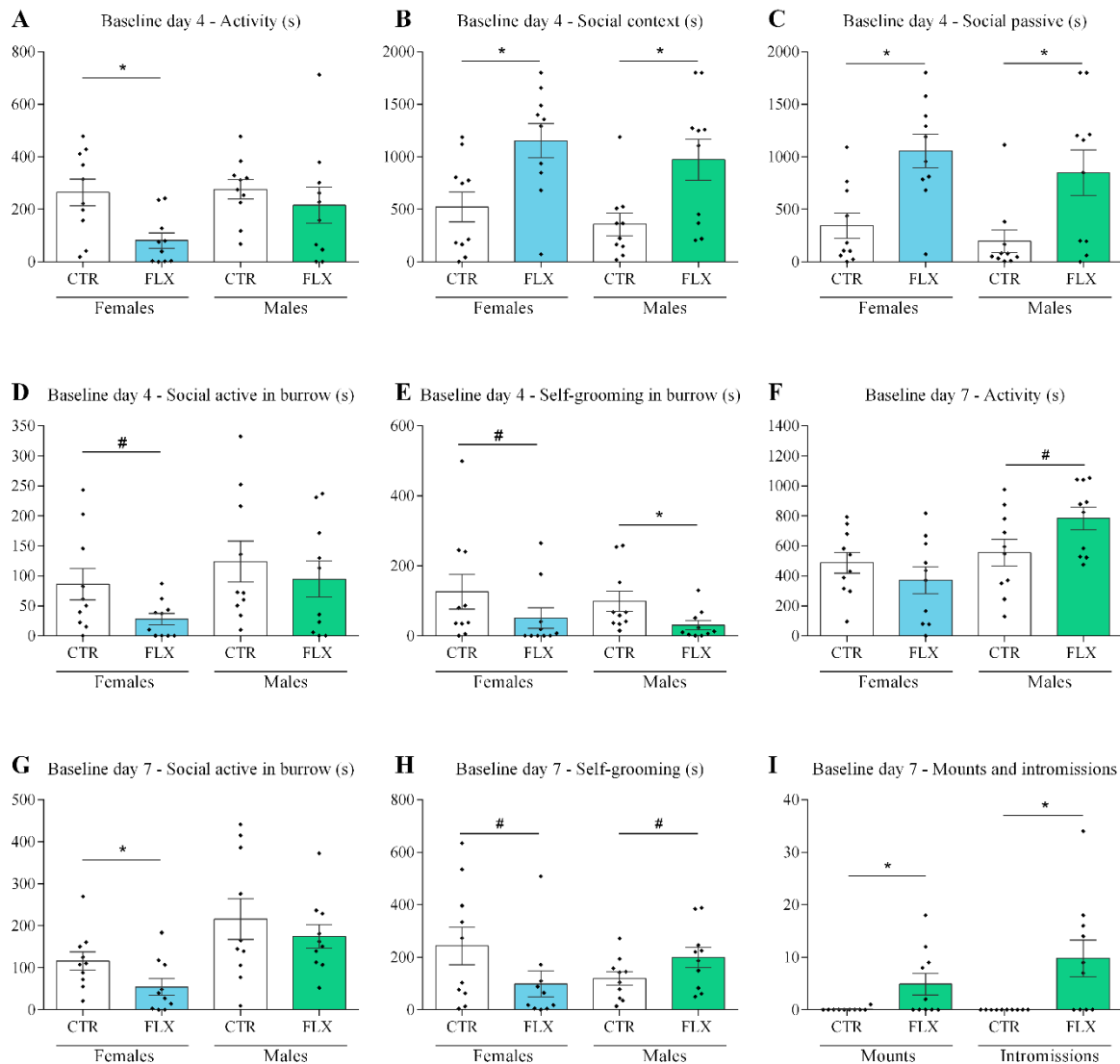
On day 4, we found that FLX-females were overall less active than CTR-females ($Z = -2.495$, $p = 0.013$, $d = 1.387$, Figure 3A), an effect that was mainly caused by a decrease in nonsocial exploratory behavior in the burrow area ($Z = -2.498$, $p = 0.012$, $d = 1.403$, Figure S3A). In males, on the other hand, no behavioral differences in general activity were found.

In terms of social behavior, we first investigated the effects on all social behaviors together, meaning a combination of social passive (e.g. resting in groups) *and* active social

behaviors (e.g. sniffing and grooming behavior towards others) pooled into one parameter called “social context”. It was found that both FLX-females ($Z = -2.495$, $p = 0.013$, $d = 1.292$) and FLX-males ($Z = -2.344$, $p = 0.019$, $d = 1.236$) appear to engage in total social behavior more than CTR-rats (Figure 3B). When investigating the type of social behavior (passive versus active) in more detail, it was found that this increase in social behavior was caused by an increase in the amount of time spent on passive social behavior like resting and/or hiding in the vicinity of another rats (females: $Z = -2.873$, $p = 0.004$, $d = 1.598$; males: $Z = -2.873$, $p = 0.023$, $d = 1.191$, Figure 3C). In contrast, FLX-females, but not FLX-males, tended to spend less time on active social interactions (such as sniffing others) in the burrow area than CTR-females (trend: $Z = -1.828$, $p = 0.068$, $d = 0.929$, Figure 3D).

When looking at conflict behavior, even though the total amount of time that was measured in this behavior was limited, FLX-females were for a significantly shorter duration involved in conflicts in the burrow area than CTR-females ($Z = -2.097$, $p = 0.036$, $d = 0.914$, Figure S3B). This difference was not found in FLX-males. Another finding that was more pronounced in male rats during baseline measures on day 4, was that FLX-males spent less time grooming themselves compared with CTR-rats ($Z = -2.344$, $p = 0.019$, $d = 0.881$, Figure 3E). FLX-females, also groomed themselves slightly less than CTR-females, although this just missed significance (trend: $Z = -1.745$, $p = 0.081$, $d = 0.556$).

Figure 3. Behavioral baseline effects of perinatal SSRI exposure



*Figure 3. The data represents the time spent (s) on each behavior at adulthood in the seminatural environment at baseline on day 4 and 7: general activity (day 4) (A), being in a social context (day 4) (B), being socially passive (day 4) (C), social activity in the burrow area (day 4) (D), self-grooming in the burrow area (day 4) (E), general activity (day 7) (F), social activity in the burrow area (day 7) (G), self-grooming (day 7) (H), and number of mounts and intromissions (day 7) (I). All graphs show the comparison between FLX-females (n=10) and CTR-females (n=10), and/or FLX-males (n=10) versus CTR-males (n=10). Data are shown with individual data points, with the bars representing the mean ± standard error of the mean. * p < 0.05, # p < 0.1 compared with CTR-females or CTR-males.*

On day 7, the females were in menstrual proestrus due to estrogen and progesterone injections on day 5 and 7, respectively. Consequently, they became sexually receptive, which resulted in the display of sexual interactions. Given the background of this intervention, it was found that most behavioral differences present on day 4 baseline were absent on day 7 baseline.

On day 7, we actually found that FLX-females were just as (non-socially) active as CTR-females (Figure 3F). At the same time, FLX-females spent more time being passive than CTR-females ($Z=-1.268$, $p=0.023$, $d = 1.034$, Figure S3C), an effect that was mostly caused by an increase in time spent hiding instead of a difference in socially or solitary resting (as on day 4).

Although FLX-females still spent more time in a social context, this effect was no longer significant on day 7 (Figure S3D). However, a behavior that was still present on day 7 (and comparable/stronger compared to day 4) was the amount of active social behavior: FLX-females had less social interactions than CTR-females in mainly the burrow area ($Z= -2.117$, $p= 0.034$, $d = 0.996$, Figure 3G). In contrast, when we look at the amount of sexual interactions, FLX-females spent less time showing paracopulatory behavior ($Z= -2.008$, $p= 0.045$, $d = 0.351$, Figure S3E), and showed a tendency in receiving less mounts than CTR-females did (trend: $Z= -1.819$, $p= 0.069$, $d = 0.509$; Table S2). Furthermore, FLX-females were pursued by other rats for a shorter duration compared with CTR-females ($Z= -2.260$, $p= 0.024$, $d = 0.351$, Figure S3F). As a consequence, FLX-females also showed fewer lordosis responses than CTR-females (trend: $Z= -1.954$, $p= 0.051$, $d = 0.610$, Figure S3G). In terms of self-grooming, on day 7 during the baseline period, FLX-females also tended to groom themselves less than CTR-females in the burrow area (trend: $Z= -1.777$, $p= 0.076$, $d = 0.784$, Figure 3H).

Now that the females were receptive, FLX-males started to show an interesting pattern of behavior. A trend was found towards an increase in general activity in FLX-males compared to CTR-males (trend: $Z= -1.739$, $p= 0.082$, $d = 0.810$, Figure 3F), mostly seen in the open field ($Z=$

-1.752, $p = 0.08$, $d = 0.981$). This effect was most likely, but not solely, caused by an increase in the amount of time FLX-males spent pursuing other rats in the open field compared with CTR-males (trend: $Z = -1.757$, $p = 0.079$, $d = 0.952$, Figure S3H). This pursuing behavior was necessary for the sexual interactions: FLX-males mounted ($Z = -2.097$, $p = 0.036$, $d = 1.048$) and intromitted ($Z = -2.796$, $p = 0.005$, $d = 1.253$) more often than CTR-males (Figure 3I). At the same time, the sexual behavior induced the display of postcopulatory self-grooming, immediately explaining the higher amount of both the postcopulatory self-grooming ($Z = -2.484$, $p = 0.013$, $d = 1.368$, Table S2) and trend in higher amount of self-grooming (trend: $Z = -1.663$, $p = 0.096$, $d = 0.790$, Figure 3H) in FLX-males compared with CTR-males. As a logical consequence of the higher activity, there was also a trend that FLX-males were less passive than CTR-males (trend: $Z = -1.814$, $p = 0.07$, $d = 0.930$, Figure S3C).

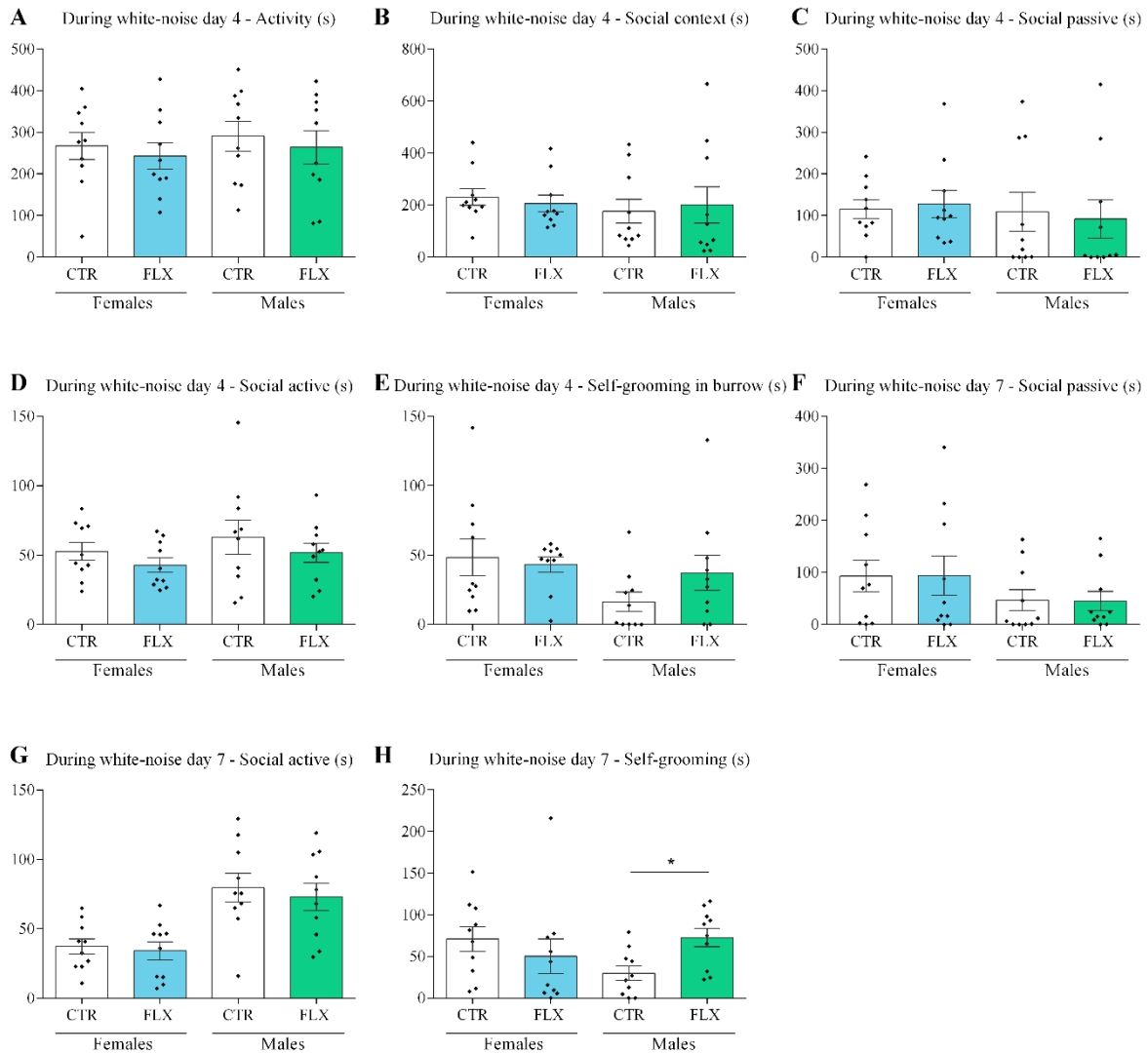
3.2 Behavior during white-noise exposure

Secondly, we were interested in whether perinatal SSRI exposure affects coping with a stressor. Therefore, we exposed the rats to a 10-minute white-noise stressor and measured their behavioral responses during this period. Interestingly, we found that FLX-rats responded in a similar way to the white-noise as CTR-rats on day 4 (Figure 4A-E). The only interesting finding was that FLX-males changed from grooming themselves slightly less than CTR-males in the period before the stressor to grooming themselves now more during the exposure to the white-noise, but they still did not groom themselves more than CTR-males ($Z = -1.379$, N.S.). FLX-females, on the other hand, groom themselves equally compared with CTR-females (Figure 4E). No significant differences were found between the amounts of time spent freezing upon white-noise exposure (Table S2).

On day 7, we found that again that FLX-rats responded similarly to the white-noise exposure as CTR-rats (Figure 4F/G and Table S2), except for self-grooming behavior. FLX-males groomed themselves extensively more than CTR-males ($Z = -2.571$, $p = 0.01$, $d = 1.351$, Figure 4H). No difference in self-grooming was found in the females. In addition, no significant differences were found in freezing behavior (Table S2).

In terms of sexual activity-related behavior, the only relevant different was that FLX-females were still pursued less by other rats during the white-noise episode than CTR-females ($Z = -2.097$, $p = 0.036$, $d = 0.983$, Table S2).

Figure 4. Behavioral effects of perinatal SSRI exposure during white-noise exposure



*Figure 4. The data represents the time spent (s) on each behavior at adulthood in the seminatural environment during white-noise exposure on day 4 and 7: general activity (day 4) (A), being in a social context (day 4) (B), being socially passive (day 4) (C), social activity (day 4) (D), self-grooming in the burrow area (day 4) (E), social passive (day 7) (F), social activity (day 7) (G), and self-grooming (day 7) (H). All graphs show the comparison between FLX-females (n=10) and CTR-females (n=10), and/or FLX-males (n=10) versus CTR-males (n=10). Data are shown with individual data points, with the bars representing the mean ± standard error of the mean. * $p < 0.05$*

3.3 Behavior after the stressor

At last, we were interested in whether FLX-rats are responded different to a stressor compared with CTR-rats and investigated the behavior after the white-noise exposure. We looked at whether behavioral differences from baseline persisted after a stressful event, and/or whether new behavioral variances appeared after the stressor between FLX- and CTR-rats. Therefore, we observed the behavior on day 4 and day 7 at 15:10 h after the stressor, during the dark phase. Our results of day 4 showed that the differences in behavior found at baseline were attenuated after the stressor (Figure 5A-D). We found no differences between FLX-males and females and CTR-males and females in their general activity or their behavior in social context. In terms of active social interaction, FLX-females still seem to spend less time interacting socially compared with CTR-females, but the effect was no longer significant ($Z = -1.436$, N.S., Figure 5D).

However, when we looked in more detail into the time spent in social context and calculated the percentage of time spent in social passive behavior before and after the stressor, we found that FLX-females actually responded differently to the stressor than CTR-females. As shown in Figure 6, both CTR-females and FLX-females seem to have rats who do not behave differently after a stressor, however, while part of the CTR-females increase the percentage of social resting, a large part of FLX-females clearly decrease their percentage in a social environment (and start resting more solitarily). Although the group was divided, the effect of FLX-females was significantly different ($Z = -2.041$, $p = 0.041$, $d = 0.845$). CTR-males and FLX-males did not show such a different pattern in social passive behavior before and after the stressor.

However, in males, the stressor did seriously affect the self-grooming behavior of FLX-males. We found that the increase in self-grooming behavior that was found during the white-noise period in FLX-males, was strengthened during the period after the stressor. After the

stressor, FLX-males significantly groomed themselves longer than CTR-males ($Z = -2.519$, $p = 0.012$, $d = 1.670$, Figure 5E). FLX-males also groomed themselves significantly longer after the stressor compared with baseline ($Z = -3.141$, $p = 0.002$, $d = 2.156$, Figure 7C-D). FLX-females now groomed themselves in a level similar to CTR-females. However, when baseline and after stressor were compared, FLX-females also groomed themselves significantly longer ($Z = 2.324$, $p = 0.02$, $d = 1.122$, Figure 7A-B). Lastly, it should be mentioned that FLX-males, compared with CTR-males, were observed freezing for a longer total period of time after white-noise exposure when in the open field ($Z = -2.163$, $p = 0.031$, $d = 0.783$, Table S2). Although brief as that was, it is still interesting because four FLX-males show this behavior, whereas none of the CTR-males in the open field were seen freezing.

On day 7, we found again no behavioral differences between FLX-rats and CTR-rats after exposure to the stressor (Figure 5F-H, Table S1). The only difference we found was the increased levels of self-grooming in FLX-males on day 7 after the stressor, although it did not reach significance in the amount of time spent on it, but only in the number of self-groom episodes ($Z = -2.091$, $p = 0.037$, $d = 0.987$, Table S2). In addition, FLX-males continued copulating: FLX-males had more intromissions than CTR-males ($Z = -2.163$, $p = 0.031$, $d = 0.799$, Figure 5I), although this effect was caused by only 3 copulating males. However, interestingly, the copulatory behavior were now mostly performed in the burrow area instead of in the open field. Indicating that the stressor affected the location in which copulation takes place. FLX-females, on the other hand, now spent an equal amount of time on sexual activity as CTR-females (Table S2).

Figure 5. Behavioral effects of perinatal SSRI exposure after a stressor

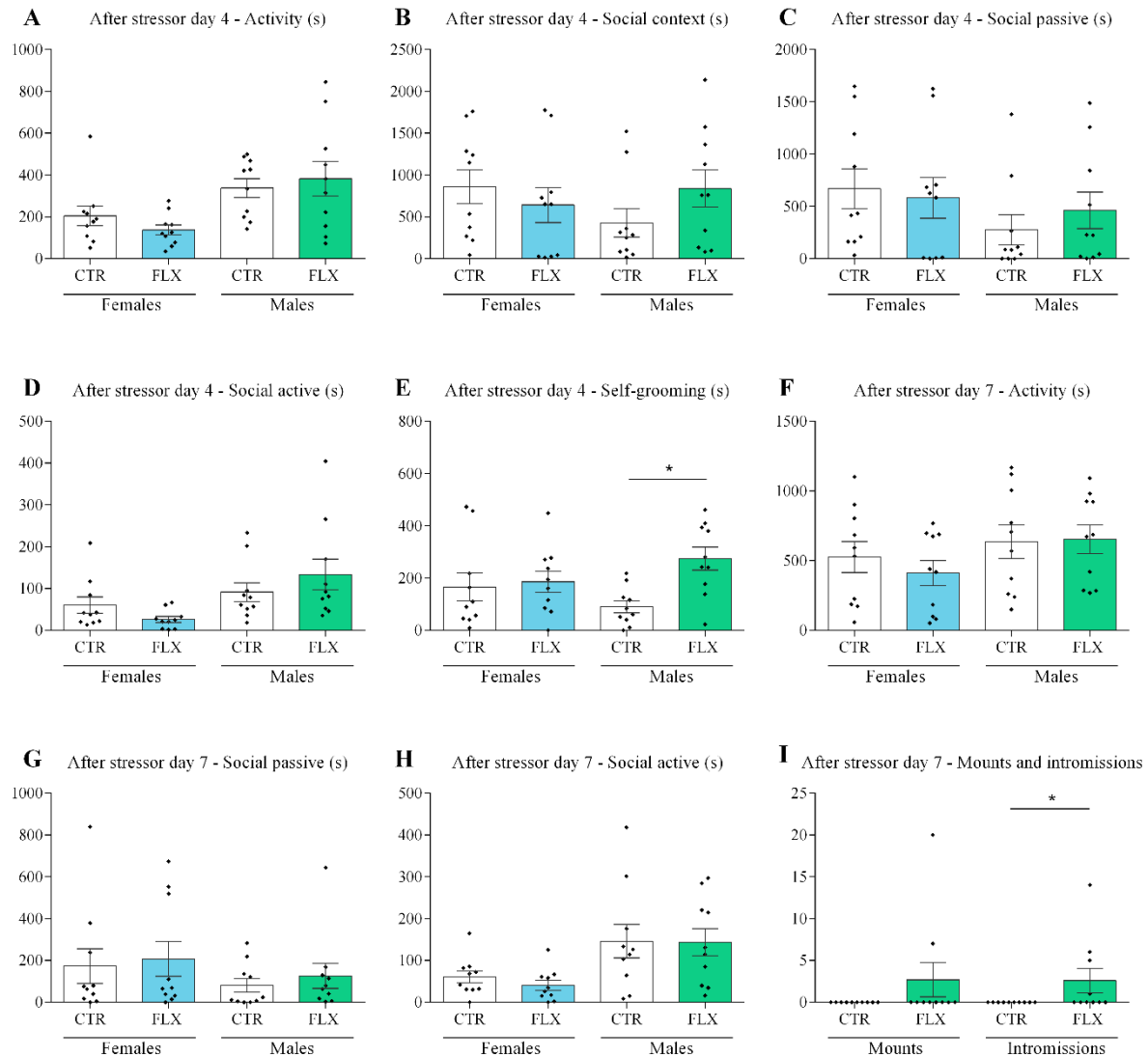
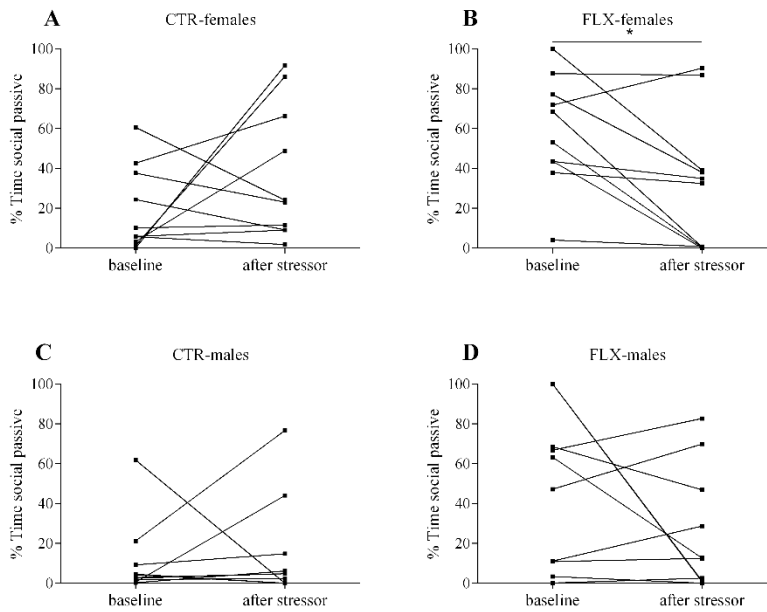


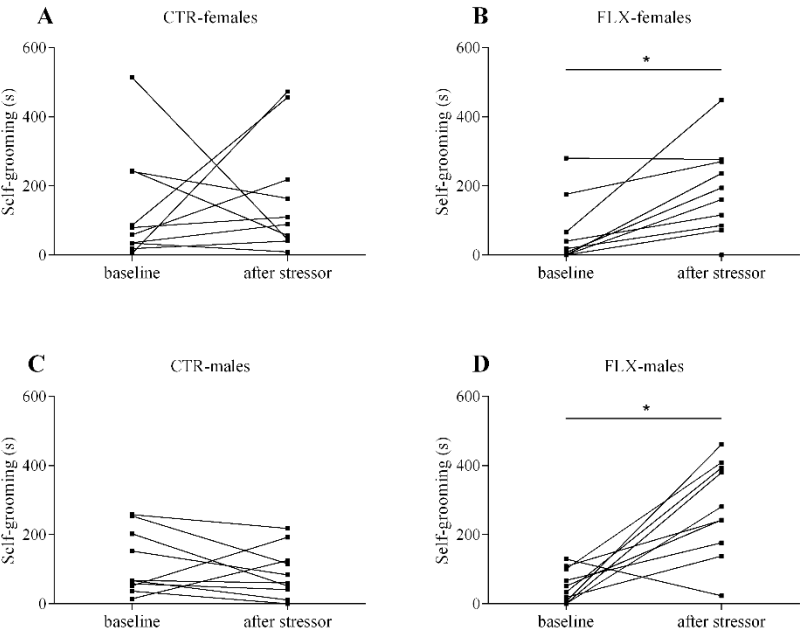
Figure 5. The data represents the time spent (s) on each behavior at adulthood in the seminatural environment after a white-noise exposure on day 4 and 7: general activity (day 4) (A), being in a social context (day 4) (B), being socially passive (day 4) (C), social activity (day 4) (D), self-grooming in the burrow area (day 4) (E), general activity (day 7) (F), social passive (day 7) (G), social active (day 7) (H), and number of mounds and intrusions (day 7) (I). All graphs show the comparison between FLX-females ($n=10$) and CTR-females ($n=10$), and/or FLX-males ($n=10$) versus CTR-males ($n=10$). Data are shown with individual data points, with the bars representing the mean \pm standard error of the mean. * $p < 0.05$

Figure 6. Difference in percentage of time spent on being social passive



*Figure 6. The data represents the percentage of time rats spent on being socially passive. All graphs show the comparison between baseline and after stressor of CTR-females (n=10, A), FLX-females (n=10, B), CTR-males (n=10, C), and FLX-males (n=10, D). Data are shown in individual data points, with the lines connecting the same individuals at baseline and after stressor. * $p < 0.05$*

Figure 7. Difference in time spent grooming on baseline and after stressor



*Figure 7. The data represents the time spent (s) on grooming themselves at adulthood in the seminatural environment on day 4: CTR-females (A), FLX-females (B), CTR-males (C), and FLX-males (D) Data are shown in individual data points, with the lines connecting the same individuals at baseline and after stressor. * $p < 0.05$*

4. Discussion

In the present study we sought to study the effects of perinatal SSRI-exposure on neurobehavioral outcomes in adult offspring using a seminatural environment, allowing us to control environmental factors and observe the full behavioral repertoire of the animals in a more naturalistic setting.

Our findings indicate that perinatal SSRI exposure can induce behavioral adaptations. Rats that were exposed to SSRIs during early development show at baseline a lower general activity at adulthood than control rats, which was mostly explained by a decrease in nonsocial exploration. In terms of social behavior, our data surprisingly showed that fluoxetine-exposed rats seek more social contact than control rats. This increased sociability, however, is a result of more passive behavior performed in a social context in both males and females. In contrast, female rats that were perinatally exposed to SSRIs tended to show less active social behaviors than control females.

When exposed to a stressful event, presented as white-noise, all rats responded similarly, resulting in an attenuation of the pre-stressed found alterations. However, when the behavior following the stressor was investigated in more detail, it was found that FLX-rats changed preference from resting in groups to more solitary resting, whereas control rats actually started to seek a more social (passive) environment. In addition, FLX-males started to self-groom themselves extensively more than before presentation of the stressor, even more than control rats. The FLX-males also showed increased freezing behavior in the open area compared to control rats.

It should be mentioned that most of the behavioral differences were found on day 4 when the females had not received any hormonal treatment and were sexually non-receptive, while no differences were found on day 7 when the females were hormonally primed and sexually

receptive. At first sight, this could indicate that the hormonal state of the females plays an important role in the expression of behavioral effects of perinatal SSRI exposure. However, an alternative explanation could be found in the fact that the females are now in their behavioral estrus and receptive for sexual interactions. Both males and females could thus be occupied by the opportunity to copulate and thereby show normal behavioral outcomes. If so, the lack of effects due to perinatal SSRI exposure during proestrus in females does not necessarily have to be a result from the hormones themselves.

4.1 Social behavior

4.1.1 Social behavior at baseline

We found that both FLX-males and FLX-females spent passive moments more often in the company of another rat (social resting) compared with CTR-rats. When both the passive and active behavior performed in a social context are analyzed as total social behavior, we found (on day 4) that fluoxetine exposure during development induced a phenotype in adulthood in which the rats are more social than control rats. This finding is in line with a recent study by Gemmel et al. that similarly treated dams with 10 mg/kg fluoxetine throughout most part of pregnancy and until weaning of the pups. Adult females from fluoxetine treated dams increased their social investigation time with another female, while adult males increased their play behavior (Gemmel et al., 2019). Furthermore, Ko et al. found that injecting male rat offspring directly with fluoxetine from PND0-4 increased their sniffing, contact and total interaction behavior with a conspecific when adult (Ko et al., 2014). In contrast, a study by Olivier et al. showed that prenatal SSRI exposure in male rats decreased the amount of time spent on social exploration behavior measured by sniffing and grooming others (Olivier et al., 2011b). Furthermore, we recently showed that fluoxetine treatment from G0 till PND21 resulted in decreased social interaction in

male but not female rats (Houwing et al., 2019b). Likewise, postnatal SSRI exposure affected social exploration time in social preference tests in which the amount of exploration time to a conspecific was compared with the time spent sniffing a novel object. Male and female offspring (postnatally treated with SSRIs) show decreased conspecific exploration compared with novel object exploration at both juvenile and adult age (Khatri et al., 2014; Rodriguez-Porcel et al., 2011; Simpson et al., 2011; Zimmerberg and Germeyan, 2015). Similarly, the majority of studies studying specifically social play behavior in rats found a decrease in social play as a result of early SSRI exposure (Khatri et al., 2014; Olivier et al., 2011b; Rodriguez-Porcel et al., 2011; Simpson et al., 2011). However, we did not study the highly playful juvenile rat but social interaction at adulthood and we barely observed play behavior at our chosen time points. In the present study, we found a tendency towards decreased active social behavior in FLX-females, but not in males. Since male rats are consistently less socially active in all the other studies, the fact that we were unable to replicate this finding, can most likely be ascribed to the test setting. Our study used a seminatural environment, which allowed us to study all behaviors expressed by the rats at the same time, meaning that the rats have the freedom to perform any behavior at any time point they want. One should also note that the basal behavior was observed at day 4, when exploration behavior was reduced (compared with day 0), and rats were no longer unfamiliar to each other. This might have influenced the findings in the present study as well. Differences of acute novel social interactions may still exist and this remains to be investigated. In a study of Gemmel et al 2017, it was shown that social play behavior in juvenile rats exposed to fluoxetine during development was increased when paired with an unfamiliar partner, while they found no differences in social play when interacting with their siblings (Gemmel et al., 2017). These data may confirm the theory that novel acute social interactions may have a different outcome when

comparing the already established social interactions such as the observations in the seminatural environment on day 4 with social interactions with siblings in their home cage.

4.1.2 Social behavior after stressor

With the additional exposure to a 10-minute white-noise stressor within this environment, we were able to investigate the acute and long-term behavioral responses to this novel and stressful stimulus, and the behavioral consequences afterwards. At first it seemed that the alterations in social behavior disappeared, but after a more detailed analysis, we found that FLX-rats actually respond differently to the stressor than CTR-rats. While FLX-rats were significantly longer passive in a social context at baseline levels compared with CTR-rats, the FLX-females started to rest less in groups (Figure 6) and more solitarily after the stressor. CTR-females, on the other hand, started to rest more in a social context. This suggests that FLX-females have the opposite response in a stressful situation than CTR-females. More research is needed to find out whether this effect is only temporary, will sustain or exacerbates over time, and whether this is alteration is advantageous or disadvantageous before serious conclusions can be drawn.

4.2 Other responses to stressor

During the actual period of white-noise exposure, both CTR- and FLX- rats were more generally active and showed more freezing behavior than baseline. However, overall our data showed that FLX-rats did not differ in their behavior from CTR-rats during the white-noise exposure. Despite the slight increase in the occurrence of freezing behavior, all rats spent the same amount of time freezing. Other studies, on the other hand, have shown that rats exposed to SSRIs during early development responded with exaggerated freezing (or sometimes measured as immobility time) to a novel tone compared with control rats (Khatri et al., 2014; Rodriguez-

Porcel et al., 2011; Simpson et al., 2011). In addition, this increase in immobility lasts longer in FLX-rats than in CTR-rats (Rodriguez-Porcel et al., 2011), suggesting that early developmental SSRI exposure induces hyperreactivity towards a novel auditory stimulus. Our data, however, does not confirm these findings. In addition, it showed that the rats in the seminatural environment, instead of showing a freezing response, started to explore and run through the burrow area more. During the 10 minute of white-noise exposure, the rats actually spent the relative same amount of time running and exploring the burrow as during the 30 minute baseline period (about a 3-fold increase). This increase in general activity is most likely a stressful response to the white-noise. The lack of effect on freezing behavior in our paradigm, on the other hand, suggests that rats respond differently to novel auditory stimuli in a seminatural environment than in a small test setting. One explanation could be that the social environment creates a kind of social buffering: the presence of familiar conspecifics have positive comforting effects in stressful situations (Kiyokawa et al., 2014; Terranova et al., 1999). Another or additional explanation, however, could again be found in the fact that rats can express all kind of behaviors in a seminatural environment, which is at the same time their home cage and test environment. While freezing is the most logical behavior in a small test set-up in response to a stressor, this behavior is not needed in large and familiar living spaces where one could just as well escape from the stressor or danger by walking away. Whatever the reasons are behind the lack of freezing behavior, our data clearly showed that FLX-rats do not respond differently to a stressor, in terms of freezing or exploratory behavior, compared with CTR-rats.

4.3 Stress-coping behavior

Simultaneously, another important change in response to the stressor was observed: a difference in stress-coping behavior in FLX-males when compared with CTR-males. While FLX-

males on day 4 groomed themselves significantly less than CTR-males at baseline, they started to self-groom more during, but especially after, the white-noise exposure (Figure 7). FLX-females also groomed themselves relatively more after the stressor, but the time spent on this behavior was not different from CTR-females. As discussed in (Smolinsky et al., 2009), grooming is an important behavior observed in many species serving several functions. Beyond the most obvious purpose of hygiene, grooming is also performed for stimulation of the skin, thermoregulation, chemo-communication, social interaction, de-arousal, and stress reduction (Sachs, 1988; Spruijt et al., 1992; Terry, 1970). In rodents, this grooming behavior is rather patterned and starts with licking of the paws, followed by washing the nose and face, the head, the body, the legs, and finally licking the tail and genitals (Fentress, 1988; Smolinsky et al., 2009). In addition, grooming is highly sensitive to various stressors, psychotropic drugs and genetic manipulations, making it an important player in behavioral adaptation to stress, including stress-coping and de-arousal (Choleris et al., 2001; Dunn et al., 1987; Spruijt et al., 1992). In fact, grooming can be interpreted as a typical displacement behavior in which an animal is in conflict to perform two or more different behaviors and where the response is a displacement activity that is usually unrelated to the competing behaviors. This stress-induced displacement grooming, however, is ethologically different from low-stress comfort grooming, indicating that the amount of grooming behavior by itself is insufficient as a measure for stress. Interestingly, differences in grooming patterns in low and high stress situations have been studied. Whereas low-stress comfort grooming occurs spontaneously as a transition between rest and activity, and usually follows an uninterrupted pattern of the order described above, high stress levels induce more frequent and rapid short bouts of interrupted less patterned activity of self-grooming (Kalueff and Tuohimaa, 2004, 2005). These differences in grooming pattern, or microstructures, could be used as indicators for different neuropsychiatric disorders (Kalueff et al., 2016): e.g. obsessive compulsive behavior

and autistic phenotypes could be related with high locomotor, but rigid patterned grooming, while anxiety would be represented by high locomotor, but more flexible patterning. Depression, on the other hand, could result in a self-groom microstructure of low locomotor activity with a slight patterned grooming (Kalueff et al., 2016).

Unfortunately, due to the fact that our seminatural environment is rather large, our video images did not have the right resolution to study the self-groom patterns in more detail. Still, the difference in self-groom behavior before and after the stressor makes it plausible to believe that the rats performed different patterns of self-grooming reflecting less comfort/hygiene grooming at baseline, compared with higher levels of stress-coping grooming after the stressor. Our baseline data is in line with a previous finding in which perinatal SSRI exposure reduced the time in which males groomed themselves during a social behavior test (Olivier et al., 2011b). At the same time, the increased levels of self-grooming coincide with the finding that FLX-rats show increased burying behavior in a marble burying test which is used to study repetitive and perseverative behavior (Sprowles et al., 2017). As a result, we hypothesize that perinatal SSRI exposure changes the stress-coping mechanisms in male rats at adulthood after the exposure to stressors. Future research should clarify whether the higher activity of grooming behavior reflects in the direction of anxiety-related versus repetitive compulsive self-grooming.

4.4 Aggressive behavior

In the seminatural environment, many more behaviors can be explored such as aggressive and sexual behaviors. Previous studies have shown that perinatal SSRI exposure increases aggressive behavior in adult male mice (Kiryanova and Dyck, 2014; Svirsky et al., 2016). However, we recently showed that fluoxetine treatment during gestation and the postnatal period reduced the offensive behavior of male rats in a resident-intruder test set-up (Houwing et al., in

preparation). In the present study, however, FLX-males spent the same amount of time in conflict situations as CTR-males, while FLX-females seem to show less conflict behaviors on day 4. We would, however, not dare to draw any serious conclusions based on this observation, because on average the rats do not spend more than 40 seconds on this agonistic behavior. Wistar rats are known to show low levels of aggressive behavior in general (Koolhaas et al., 2013), and in our seminatural environment set-up the rats do not really have to compete for resources. Drinking water and food pellets were available ad libitum, and even during the period of behavioral estrus there were enough receptive females available for mating. It is, therefore, fair to say that our experimental design was not sufficient for the exploration of aggressive encounters.

4.5 Sexual behavior

Also in terms of sexual behavior, our set-up had its limitations. Although we found an increase in copulatory behaviors in FLX-males, previous studies have shown conflicting results of early life SSRI exposure on sexual behavior. Postnatal fluoxetine exposure has been shown to decrease the amount of mounts, intromissions and ejaculations, just as reducing the level of sexual motivation in male rodents (Gouvea et al., 2008; Harris et al., 2012; Rayen et al., 2013; Rodriguez-Porcel et al., 2011). Prenatal SSRI exposure, on the other hand, did not affect male copulatory behavior (Cagiano et al., 2008; Olivier et al., 2011b). In a study we performed before, male rats exposed to fluoxetine during the whole gestational and postnatal period (until weaning) displayed a reduction in the number of mounts compared with control males, but only when the males were sexually experienced (Houwing et al. in preparation). In the FLX-females of the present study, we found a slight decrease in sexual behavior compared with CTR-females. Other studies, however, found a stimulatory effect on paracopulatory and receptive behaviors of postnatal fluoxetine exposure (Rayen et al., 2014). One could argue that the timing of the SSRI

exposure could explain our differences in results, but a better explanation could be found in the fact that we only observed 30 minutes twice. A study by Chu and Agmo performed in the seminatural environment taught us that the behavioral estrus of female rats can last up to eleven hours, with an average of 7 hours (Chu and Agmo, 2014). During this whole period, male and female rats continue to participate in copulatory behavior until the estrus period ends (Chu and Agmo, 2014, 2015). Male rats seem to copulate in copulatory bouts, defined as the time between the initial mount or intromission and the beginning of a period of sexual inactivity lasting for more than 60 min. When males copulate with naturally cycling females, they have on average about 4 ± 1 bouts during the time they are in the seminatural environment. No such detailed studies have been performed in the seminatural environment with ovariectomized and hormonally primed females, but we can assume that males will in this case copulate in bouts as well. This indicates that we might have observed a time slot in our experiment in which most of the CTR-males might coincidentally have been in a break between the copulatory bouts, whereas six out of ten FLX-males were observed within their copulatory bout. As a consequence, it would be very interesting to investigate the differences in behavioral patterns between FLX-rats and CTR-rats during the behavioral estrus period in more detail. This interesting data, however, would be quite substantial, and therefore better suitable for a separate manuscript.

4.6 Affective behavior

In our seminatural environmental approach, we cannot directly relate certain behaviors to the traditional tests, but an indication of anxiety in the seminatural environment might be reflected by visiting the open area less and by more freezing in response to a white-noise stressor. Our results indicated that FLX-rats were present in the open area just as long as CTR-rats, also after the stressful white-noise exposure. In addition, we only observed a slight increase in

freezing after the stressor when FLX-males visited the open area, caused by 4 out of 10 FLX-males. If these parameters would be a measure of anxiety-related behavior, it suggests that perinatal SSRI exposure does not or slightly increases the risk for anxiety-like behavior in adulthood. As mentioned before, the lack of clear anxiety-related behavior could also be explained by the social environment in which our rats were housed. The anxiety traits could be possibly suppressed in a more natural situation in which more behavioral escapes are an option, but come to the surface when exposed to an unnatural unfamiliar situation, or when assessed in acute stressful situations.

4.7 The translational value of the seminatural environment

Altogether, we believe that the seminatural environment is a good approach to study the effects of perinatal SSRI exposure (and other interventions) on naturally expressing behaviors. As mentioned before, the advantage of the seminatural environmental approach is that one can study a wide variety of behaviors at the same time, but in addition one can relate this behavior with other behaviors (e.g. sexual, aggressive, locomotor, and freezing) that are performed within the same setting/experiment. This is on one hand beneficial to the interpretation of the behavioral changes, because it provides additional information about the context of the behaviors, and on the other hand it permits to study several traits of psychiatric disorders at once in a natural situation. To give an example, the seminatural environmental approach allows for exploring several phenotypes often experienced by depressive persons, like reduced general activity, lack of interest in the environment, and limited social contact (social withdrawal). Before someone can be diagnosed with depression, the patient should have first of all characteristics of several traits corresponding to the disorder. But at the same time, these symptoms should cause significant distress or impairment in social, occupational, or other important areas of functioning, meaning in

daily life. The seminatural environmental approach allows us to evaluate this aspect as well. Therefore, we believe that the seminatural environment is a valuable test set-up, and has additional advantages compared with the traditional test methods and perfectly suits to study the behavioral outcomes due to SSRI treatment during development.

4.8 Limitation of this study

In the present study, we investigated the effects of fluoxetine exposure in offspring from healthy dams. However, in humans SSRI treatment during pregnancy and lactation is only used in mothers with psychopathologies. Even though exposing offspring from healthy dams to fluoxetine is of use to dissociate the effects of the SSRI from the maternal depression, looking at SSRI exposure in offspring from stressed dams would be more clinically relevant. We recently showed that fluoxetine treatment in healthy dams resulted in reduced social play behavior in male and female offspring (Houwing et al., 2019b), while male, but not female, offspring from an animal model of maternal vulnerability (Houwing et al., 2019a) showed reduced juvenile play behavior similar to offspring from fluoxetine treated healthy dams. Other studies showed that perinatal fluoxetine treatment can prevent reductions in rat juvenile social play behavior caused by pre-gestational maternal stress (Gemmell et al., 2017). Also, perinatal SSRI exposure in healthy dams resulted in reduced copulatory behaviors in male offspring, while male offspring from stressed dams were unaffected (Rayen et al., 2013). Interestingly, SSRI exposure in female offspring facilitated copulatory behaviors, regardless of maternal stress (Rayen et al., 2014). Thus, using an animal model of maternal depression and/or stress has an added value for future studies investigating effects of perinatal fluoxetine exposure in the seminatural environment.

5. Conclusion

Overall, we conclude that perinatal SSRI exposure causes adaptations in social and stress-coping behaviors at adulthood. FLX-females are mostly affected by reduced general activity and both males and females show altered social behavior. Exposing the animals to a stressor resulted in a different social strategy in FLX-females, and an altered stress-coping behavior in mainly FLX-males. This indicates the existence of sex differences in the responses to SSRI exposure during early development. Whether the adaptations found due to perinatal SSRI exposure are beneficial or disadvantageous remains to be investigated. We show that SSRI exposure during development can have long-lasting effects. However, the SSRIs in our study were administered to healthy dams. Using an animal model of depression instead would improve the clinical relevance. This would make the research more translational to the human situation in which only depressed mothers use antidepressants. In this study we used the seminatural environment and showed it is an excellent tool to study the behavioral adaptations caused by perinatal SSRI exposure (or other interventions) in order to provide better information of the relevance of these changes for the risk for psychiatric disorders.

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Supplementary materials

Perinatal fluoxetine exposure changes social and stress-coping behaviors in adult rats housed in a seminatural environment

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Table S1: Information dams and offspring**A: Treatment dams and cage distribution offspring**

Dams	Treatment	Number offspring	Male-Cage 1	Male-Cage 2	Female-Cage 3	Female-Cage 4	Female-Cage 5	Female-Cage 6
F1	FLX	7	OM1 OM2 OM3		OF2 OF3	OF1 OF4		
F2	FLX	9	OM5 OM6 OM7	OM4 OM8	OF5 OF8	OF6 OF7		
F3	CTR	13	OM9 OM12	OM10 OM11	OF12 OF16	OF9 OF15	OF11 OF14	OF10 OF13 OF17
F4	CTR	6	OM13 OM14 OM15		OF18 OF19 OF20			
F5	FLX	none						
F6	FLX	none						
F7	CTR	none						
F8	CTR	15	OM16 OM17	OM20 OM21 OM25	OF21 OF23	OF22 OF24 OF25		
F9	FLX	8	OM29 OM30 OM31		OF27 OF29	OF26 OF28 OF30		
F10	FLX	dead						

Abbreviations: M = male, F = female, CTR = methylcellulose, FLX=fluoxetine, OM = male offspring, OF = female offspring

B: Experimental design seminatural experiment

Colony	Male 1	Male 2	Male 3	Male 4	Female 1	Female 2	Female 3	Female 4
SNE1	OM1	OM5	OM9	OM13	OF2	OF5	OF12	OF21
SNE2	OM2	OM6	OM12	OM16	OF3	OF8	OF13	OF22
SNE3	OM3	OM7	OM15	OM17	OF1	OF6	OF15	OF24
SNE4	OM4	OM11	OM21	OM29	OF4	OF9	OF19	OF27
SNE5	OM8	OM10	OM20	OM30	OF7	OF14	OF20	OF26

Abbreviations: SNE = seminatural environment, OM = male offspring, OF = female offspring

Results SR1: behavior before, during and after the white-noise stressor

Baseline behavior (day 4 and 7 combined)

In our first analysis, we combined the behavioral data of both day 4 and day 7 before, during and after the stressor as one measure to investigate the general behavioral differences between CTR-rats and FLX-rats. This analysis does not take the hormonal state of the female into account, and is thus most similar to the natural situation in which females can be in different phases of their estrous cycle.

At baseline levels (before the stressor), the data analysis revealed that FE-male ($Z = -1.965$, $p = 0.049$, $d = 1.141$) and FE-female rats ($Z = -2.646$, $p = 0.008$, $d = 1.444$) spent longer in a social context than CTR-rats (Figure S1A). This effect can be mainly explained by an increased time spent resting or hiding in close vicinity to another rat (social passive: females $Z = -2.948$, $p = 0.003$, $d = 1.691$, males $Z = -1.965$, $p = 0.049$, $d = 1.140$; Figure S1B). Interestingly, when the active social behavior was investigated, it was found that only FLX-females displayed shorter social interactions than CTR-females ($Z = -1.966$, $p = 0.049$, $d = 1.066$), especially in the burrow area ($Z = -2.496$, $p = 0.013$, $d = 1.359$, Figure S1C), while the active social behavior between FE- and CTR-males was not different.

In terms of general activity without a direct social factor, it were again the FLX-females (and not the FLX-males) who showed less activity than CTR-females in mostly the burrow area ($Z = -2.797$, $p = 0.005$, $d = 1.373$, Figure S1D). In addition, this decrease in general activity was mainly caused by the fact that FLX-females spent less time exploring the total environment in a non-social context ($Z = -2.117$, $p = 0.034$, $d = 1.153$, Figure S2A).

When looking at conflict behavior, even though the total amount of time that was measured in this behavior was limited, it was found that FLX-females were shorter involved in conflict situations than CTR-females ($Z = -3.035$, $p = 0.002$, $d = 1.332$, Figure S2B), which was mostly caused by less boxing or wrestling behavior ($Z = -2.758$, $p = 0.006$, $d = 1.148$).

Self-grooming is also an important behavior in rats. At baseline levels, FLX-females spent less time grooming themselves than CTR-females ($Z = -2.231$, $p = 0.026$, $d = 1.021$, Figure S1E), especially in the burrow area ($Z = -2.419$, $p = 0.016$, $d = 1.100$). FLX-males, on the other hand, groomed themselves for an equal amount of time as CTR-males in the total environment. However, when the time spent grooming in the open field was compared, FLX-males seemed to spend actually more time grooming themselves compared to CTR-males ($Z = -2.219$, $p = 0.026$, $d = 0.969$). This could be explained by the fact that the FLX-males also showed more sexual interactions (mounts ($Z = -2.097$, $p = 0.036$, $d = 1.048$) and intromissions ($Z = -2.796$, $p = 0.005$, $d = 1.253$, Figure S2C) than CTR-males, resulting in spending more time on postcopulatory self-grooming behavior ($Z = -2.796$, $p = 0.005$, $d = 1.209$, Figure S2D) in the open field.

Interestingly, while FLX-males seem to participate more in sexual activities, FLX-females, on the other hand, spent a shorter duration in paracopulatory behaviors ($Z = -2.008$, $p = 0.045$, $d = 0.351$, Table S1). As a consequence, FLX-females are being less pursued by the males ($Z = -2.185$, $p = 0.029$, $d = 0.366$), and thus tended to receive less mounts ($Z = -1.819$, $p = 0.069$, $d = 0.508$) and showed less lordosis responses ($Z = -1.954$, $p = 0.051$, $d = 0.610$) compared to CTR-females.

Behavior during white-noise exposure (day 4 and 7 combined)

During the 10 minutes of exposure to the white-noise stressor on day 4 and 7 combined, most of these behavioral differences found at baseline levels disappeared. FLX-rats behaved similar to CTR-rats (Figure S2E-H) and spent most of their time in the burrow area. The only difference between FLX-rats and CTR-rats were found on self-grooming behavior. FLX-males started to groom themselves extensively longer during the exposure to the stressor ($Z = -2.646$, $p = 0.008$, $d = 1.308$, Figure S1F). No significant differences were found between FLX-females and CTR-females. Though, one CTR-female

continues to show lordosis behavior during the exposure to white-noise, which resulted in a significant increase in lordosis responses compared to FLX-females ($Z = -2.164$, $p = 0.030$, $d = 0.556$, Table S1).

One might expect to find differences in the freezing behavior between FLX-rats and CTR-rats during the exposure to white-noise. However, analysis of the data revealed that no such differences were found (Table S1).

Behavior after the stressor (day 4 and 7 combined)

When the behavior after the exposure to white-noise was investigated, the data revealed that the differences in behavior during the baseline period were mostly attenuated (Figure S2I-L), except for active social and conflict behavior. FLX-females continued to show lower levels of active social behavior ($Z = -1.965$, $p = 0.049$, $d = 1.047$, Figure S2L) and conflict behavior ($Z = -1.973$, $p = 0.049$, $d = 0.591$, Table S1) compared to CTR-females.

Interestingly, one important behavior suddenly appeared during this period: allogrooming (see the discussion for an explanation of the relevance of this behavior). FLX-females tended to spend less time allogrooming other rats than CTR-females ($Z = -1.738$, $p = 0.082$, $d = 0.897$, Figure S1G). This effect was only profound in FLX-females, probably because males did in general not spend that much time allogrooming.

The effect that was found in FLX-males on self-grooming during the exposure to white-noise was maintained after the stressor was turned off. FLX-males continued to groom themselves significantly longer than CTR-males ($Z = -2.570$, $p = 0.01$, $d = 1.368$, Figure S1H). Oppositely, FLX-females seemed to self-groom to a similar level as CTR-females, whereas they groomed less at baseline level. At last, it should be mentioned that FLX-males spent more time freezing after white-noise exposure when in the open field ($Z = -1.689$, $p = 0.091$, $d = 0.375$, Table S1). Although brief as that was,

it is still interesting because four FLX-males show this behavior, whereas only one of the CTR-males in the open field were seen freezing.

Figure S1. Behavioral effects of perinatal SSRI exposure on day 4 and 7 combined

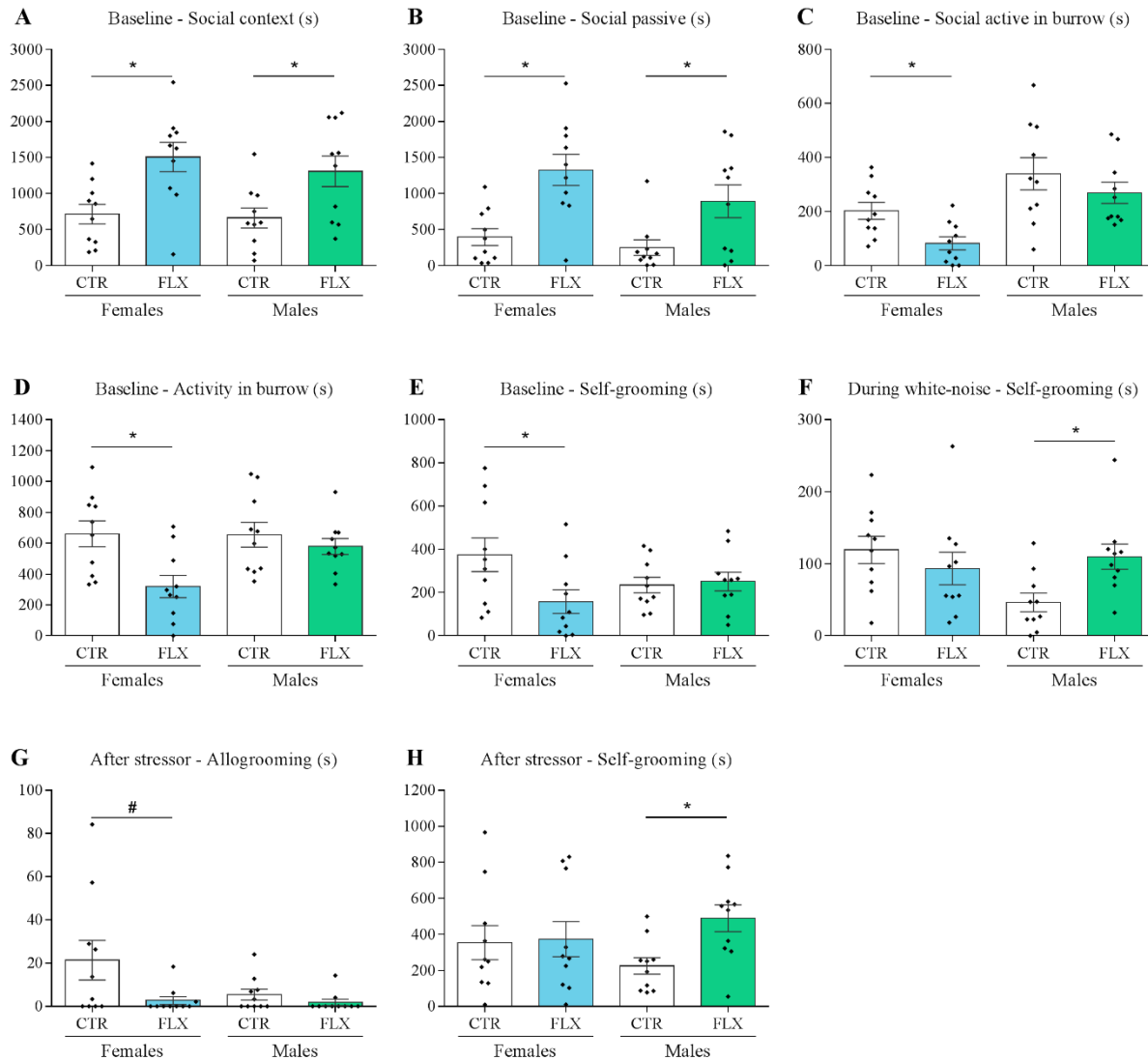
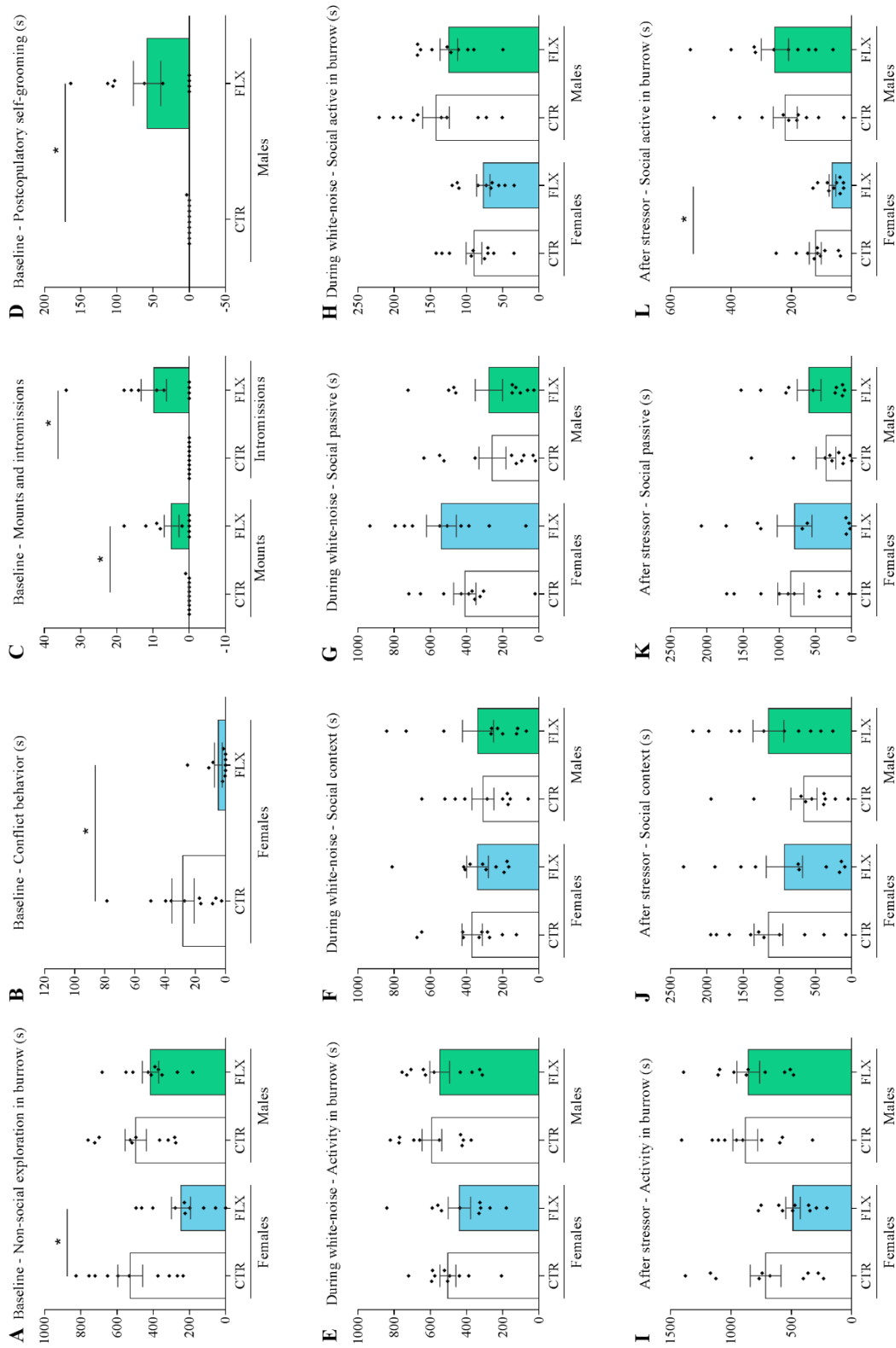


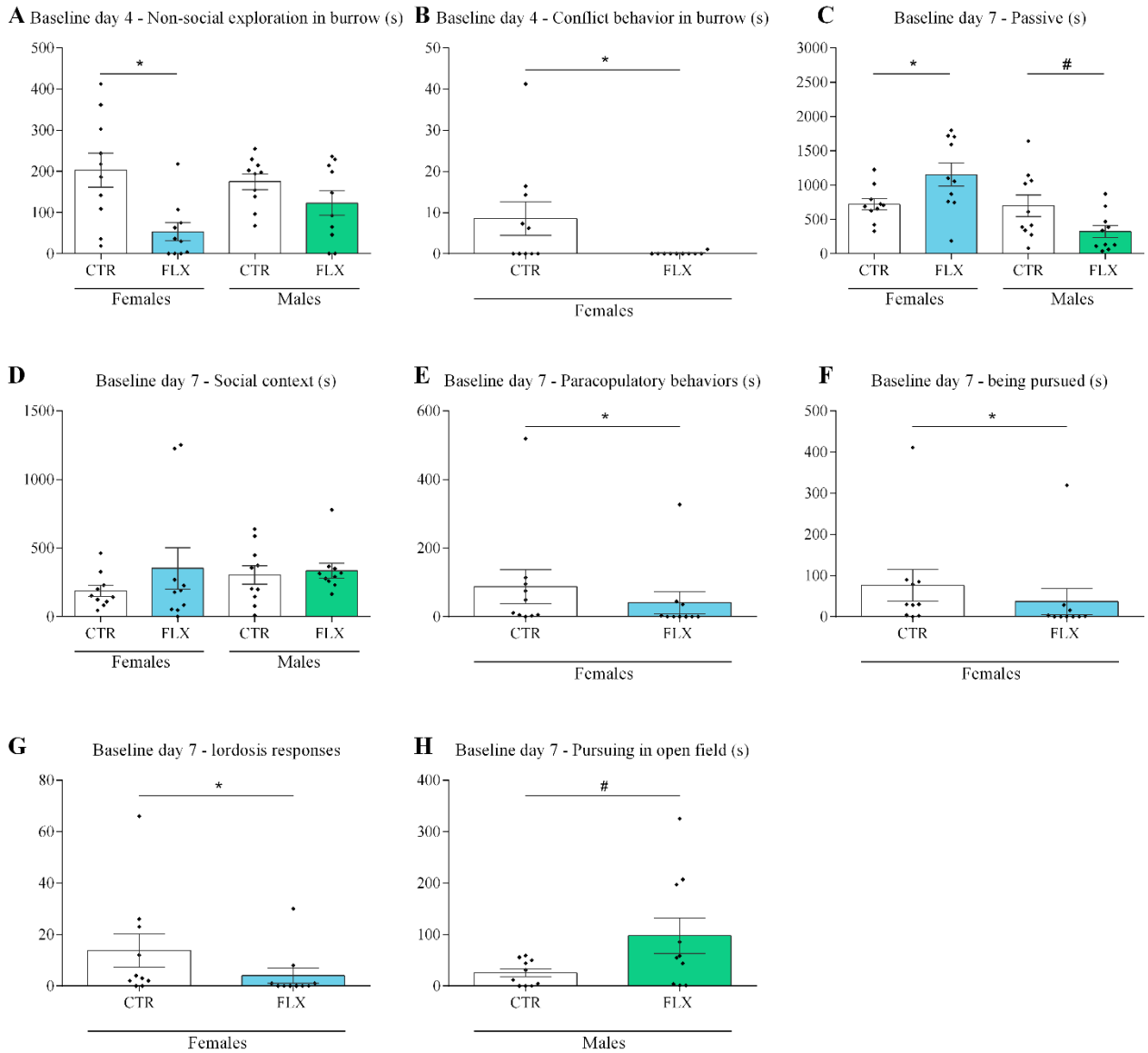
Figure S1. The data represents the time spent (s) on each behavior at adulthood in the seminatural environment on day 4 and 7 combined: baseline measure of being in social context (A), baseline measure of being socially passive (B), baseline measure social activity in the burrow area (C), baseline measure of general activity in the burrow area (D), baseline measure of self-grooming (E), self-grooming during the white-noise exposure (F), allogrooming after the stressor (G), self-grooming after the stressor (H). All graphs show the comparison between FLX-females ($n=10$) and CTR-females ($n=10$), and FLX-males ($n=10$) versus CTR-males ($n=10$). In graphs A-H, data are shown with individual data points with the bars representing the mean \pm standard error of the mean. * $p < 0.05$, # $p < 0.1$ compared to CTR-females or CTR-males. ^a $p < 0.05$, ^b $p < 0.1$ compared to baseline of the same treatment group.

Figure S2. Other behavioral effects of perinatal SSRI exposure on day 4 and 7 combined



*Figure S2. The data represents the time spent (s) on each behavior at adulthood in the seminatural environment on day 4 and 7 combined: baseline measure of non-social exploration in the burrow (A), baseline measure conflict behaviors (B), baseline number of mounts and intromissions (C), baseline measure of postcopulatory grooming (D), general activity in the burrow area during white-noise exposure (E), being in a social context during white-noise exposure (F), Being socially passive during white-noise exposure (G), social activity in the burrow during white-noise exposure (H), general activity in the burrow after the stressor (I), being in a social context after the stressor (J), being socially passive after the stressor (K), social activity after the stressor (L). All graphs show the comparison between FLX-females (n=10) and CTR-females (n=10), and FE-males (n=10) versus CTR-males (n=10). Data are shown with individual data points with the bars representing the mean±standard error of the mean. * p<0.05, # p<0.1 compared to CTR-females or CTR-males.*

Figure S3. Other baseline behavioral effects of perinatal SSRI exposure



*Figure S3. The data represents the time spent (s) on each behavior at adulthood in the seminatural environment at baseline: non-social exploration in the burrow (day 4) (A), conflict behaviors (day 4) (B), passive behavior (day 7) (C), being in a social context (day 7) (D), paracopulatory behaviors (day 7) (E), being pursued (day 7) (F), lordosis responses (day 7) (G), pursuing in open field (day 7) (H). All graphs show the comparison between FLX-females (n=10) and/or CTR-females (n=10), and FE-males (n=10) versus CTR-males (n=10). Data are shown with individual data points with the bars representing the mean \pm standard error of the mean. * $p < 0.05$, # $p < 0.1$ compared to CTR-females or CTR-males.*

Total behavior: day 7 (continued)

Behavior	Rats	Baseline						White noise						After stressor					
		Total		Burrow		Open field		Total		Burrow		Open field		Total		Burrow		Open field	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Number of mounts	CTR-females	3.9	2.2	1.1	0.9	2.8	1.8	0.5	0.3	0.5	0.3	0.0	0.0	1.9	1.1	1.9	1.1	0.0	0.0
	FLX-females	1.1#	1.1	0.1	0.1	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.8	0.8	0.8	0.0	0.0
	CTR-males	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	FLX-males	4.9*	2.0	1.1	1.0	3.8*	1.5	0.5#	0.3	0.5#	0.3	0.0	0.0	2.7	2.0	2.7	2.0	0.0	0.0
Number of intromissions	CTR-females	8.1	4.7	1.5	0.8	6.6	4.6	1.4	1.3	1.4	1.3	0.0	0.0	2.1	1.4	2.1	1.4	0.0	0.0
	FLX-females	1.7	1.6	0.0*	0.0	1.7	1.6	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.5	0.4	0.4	0.1	0.1
	CTR-males	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	FLX-males	9.8*	3.5	1.5#	0.8	8.3*	3.5	1.4	1.3	1.4	1.3	0.0	0.0	2.6*	1.5	2.5*	1.4	0.1	0.1
Number of ejaculations	CTR-females	0.2	0.2	0.0	0.0	0.2	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.0	0.0
	FLX-females	0.1	0.1	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	CTR-males	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	FLX-males	0.3	0.2	0.3	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.0	0.0
Paracopulatory behaviors	CTR-females	87.7	49.8	9.0	3.7	78.7	50.3	1.5	1.4	1.5	1.4	0.0	0.0	1.9	0.9	1.5	0.7	0.4	0.3
	FLX-females	41.1*	32.2	4.5*	4.1	36.6	32.1	0.0	0.0	0.0	0.0	0.0	0.0	2.6	1.3	0.9	0.6	1.7	1.3
	CTR-males	18.4	7.9	8.7	5.1	9.7	6.7	0.2	0.2	0.2	0.2	0.0	0.0	1.5	1.0	1.1	0.9	0.3	0.2
	FLX-males	104.3#	38.4	4.5	2.0	99.8#	38.8	1.4	1.4	1.4	1.4	0.0	0.0	3.0	1.4	1.2	0.8	1.8	1.1
Number of lordoses	CTR-females	13.8	6.5	3.5	1.6	10.3	6.4	2.2	1.8	2.2	1.8	0.0	0.0	4.7	2.6	4.7	2.6	0.0	0.0
	FLX-females	4.0#	3.0	1.0#	0.8	3.0	2.9	0.0*	0.0	0.0	0.0	0.0	0.0	1.5	1.0	1.4	0.9	0.1	0.1
	CTR-males	1.7	1.1	1.7	1.1	0.0	0.0	0.2	0.1	0.2	0.1	0.0	0.0	0.5	0.3	0.5	0.3	0.0	0.0
	FLX-males	16.1*	5.1	2.8	1.8	13.3*	5.0	2.0	1.8	2.0	1.8	0.0	0.0	5.7	3.4	5.6	3.4	0.1	0.1
Carrying nesting material	CTR-females	0.4	0.4	0.3	0.3	0.2	0.2	0.7	0.7	0.7	0.7	0.0	0.0	2.5	1.7	2.5	1.7	0.0	0.0
	FLX-females	0.2	0.2	0.0	0.0	0.2	0.2	0.0	0.0	0.0	0.0	0.0	0.0	2.4	1.6	2.4	1.6	0.0	0.0
	CTR-males	0.2	0.2	0.2	0.2	0.0	0.0	2.2	2.2	2.2	2.2	0.0	0.0	2.1	2.0	2.1	2.0	0.0	0.0
	FLX-males	0.3	0.3	0.3	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Carrying wood sticks	CTR-females	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	FLX-females	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	CTR-males	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	FLX-males	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pushing bedding	CTR-females	1.2	0.8	1.2	0.8	0.0	0.0	1.6	1.6	1.6	1.6	0.0	0.0	5.6	3.3	5.6	3.3	0.0	0.0
	FLX-females	4.6	3.0	3.9	3.0	0.7	0.6	0.0	0.0	0.0	0.0	0.0	0.0	19.8	11.3	19.8	11.3	0.0	0.0
	CTR-males	1.8	1.3	0.5	0.3	1.2	1.1	8.8	7.7	8.8	7.7	0.0	0.0	0.6	0.4	0.6	0.4	0.0	0.0
	FLX-males	3.5	2.2	3.5	2.2	0.0	0.0	0.0#	0.0	0.0#	0.0	0.0	0.0	4.3	3.1	4.3	3.1	0.0	0.0
Self-grooming	CTR-females	243.4	71.6	234.2	72.1	9.2	5.6	71.0	14.7	71.0	14.7	0.0	0.0	187.5	66.4	187.2	66.4	0.2	0.2
	FLX-females	99.0	49.0	80.5#	49.7	18.6	9.4	50.4	20.6	50.4	20.6	0.0	0.0	187.2	68.0	181.8	69.4	5.5	4.4
	CTR-males	118.8	25.2	111.9	23.7	6.9	6.0	29.9	8.7	29.9	8.7	0.0	0.0	134.1	46.0	134.1	46.0	0.0	0.0
	FLX-males	199.6#	38.1	108.9	32.8	90.6*	29.5	72.7*	11.2	72.7*	11.2	0.0	0.0	214.7	56.7	205.5	59.1	9.2#	5.3
Postcopulatory self-grooming	CTR-females	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	FLX-females	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	CTR-males	0.4	0.4	0.4	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	FLX-males	58.4*	19.0	7.0*	4.1	51.4*	19.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Drinking/eating	CTR-females	19.1	11.7	0.0	0.0	19.1	11.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	FLX-females	5.2	3.4	0.0	0.0	5.2	3.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	CTR-males	10.3	5.5	0.0	0.0	10.3	5.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	FLX-males	3.7	2.9	0.0	0.0	3.7	2.9	0.0	0.0	0.0	0.0	0.0	0.0	13.8	13.8	12.9	12.9	0.9	0.9
Freezing	CTR-females	0.0	0.0	0.0	0.0	0.0	0.0	20.4	9.7	20.4	9.7	0.0	0.0	3.8	1.4	3.8	1.4	0.0	0.0
	FLX-females	0.0	0.0	0.0	0.0	0.0	0.0	22.8	13.1	22.8	13.1	0.0	0.0	8.9	2.5	8.9	2.5	0.0	0.0
	CTR-males	0.6	0.5	0.0	0.0	0.6	0.5	63.6	11.3	63.6	11.3	0.0	0.0	9.7	2.2	9.4	2.1	0.4	0.4
	FLX-males	3.6	2.6	2.6	1.9	1.0	0.7	42.6	10.2	42.0	10.3	0.6	0.6	6.4	1.6	5.3	1.6	1.1	1.1
In opening	CTR-females	78.6	28.8	78.4	28.9	0.2	0.2	0.1	0.1	0.1	0.1	0.0	0.0	30.0	9.8	29.9	9.8	0.1	0.1
	FLX-females	37.5	15.3	36.6	15.3	0.9	0.7	0.0	0.0	0.0	0.0	0.0	0.0	35.9	16.2	35.7	16.2	0.2	0.2
	CTR-males	142.5	27.6	142.2	27.5	0.3	0.1	1.8	1.2	1.8	1.2	0.0	0.0	121.1	27.3	119.9	27.2	1.2	0.8
	FLX-males	160.7	41.8	141.5	47.0	19.2*	10.2	0.3	0.2	0.3	0.2	0.0	0.0	123.9	30.0	111.9	29.1	11.9	8.6
Rearing	CTR-females	0.5	0.3	0.0	0.0	0.5	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	FLX-females	0.7	0.4	0.0	0.0	0.7	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	CTR-males	0.5	0.5	0.0	0.0	0.5	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	FLX-males	1.1	0.5	0.0	0.0	1.1	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Being pursued	CTR-females	76.0	38.8	21.9	4.9	54.1	37.0	4.5	1.9	4.5	1.9	0.0	0.0	10.2	5.0	9.2	4.4	1.0	0.8
	FLX-females	36.8*	31.5	7.5*	4.4	29.3	27.9	0.2*	0.2	0.2*	0.2	0.0	0.0	4.3	2.3	2.1*	1.7	2.2	1.3
	CTR-males	2.3	1.0	0.7	0.5	1.7	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.5	0.0	0.0	0.7	0.5
	FLX-males	12.4*	3.9	0.3	0.2	12.1*	3.8	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.6	0.0	0.0	0.9	0.6
Being anogenitally sniffed	CTR-females	73.2	22.3	49.1	14.1	24.1	21.2	20.0	6.9	20.0	6.9	0.0	0.0	21.5	9.6	21.5	9.6	0.0	0.0
	FLX-females	26.4#	11.1	24.5	10.8	1.9	1.2	4.7*	2.5	4.7*	2.5	0.0	0.0	12.4	4.8	9.2	4.4	3.1	2.1
	CTR-males	0.3	0.3	0.3	0.3	0.0	0.0	0.1	0.1	0.1	0.1	0.0	0.0	1.8	0.8	1.8	0.8	0.0	0.0
	FLX-males	1.3	0.8	1.2	0.8	0.1	0.1	0.3	0.2	0.3	0.2	0.0	0.0	0.1#	0.1	0.1#	0.1	0.0	0.0
Being groomed	CTR-females	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.2	0.2	0.	