Antidepressant use during pregnancy

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Chapter 7

General discussion

Why, How, and What

Maternal depression and SSRI use during pregnancy

Maternal depression during pregnancy is a large public health concern[1–4]. Many women require treatment for mood disorders while they are pregnant, resulting in a 2-5% use of SSRI antidepressants in this population[5–8]. SSRIs are considered relatively safe for use during pregnancy[9], but there are concerns about potential long-term effects, particularly on the brain development of the exposed child[10]. On the other hand, untreated maternal depression is also known to affect the developing child[11]. It is therefore vital that we learn more about the contribution of maternal depression, SSRI antidepressant use, and the combination of these two factors on offspring development.

Human studies in this field are observational in nature and it is difficult to disentangle any effects of SSRI use from those caused by depression. There are also limitations to studying the brain in humans. In many respects, including in aspects of brain development, rodents are similar to humans. Rodent experiments are useful to research the underlying mechanisms of the long-term effects of exposure to maternal depressive-like symptoms and SSRIs during early development. For these and other reasons, laboratory rodents are often used as model systems to study this topic.

Aim of thesis

The overall aim of this PhD thesis was to examine potential mechanisms linking perinatal SSRI antidepressant exposure to long-term health outcomes, using a rat model of maternal depressive-like symptoms.

Experimental approach

In **Chapter 2**, previously published literature on the effects of perinatal SSRI exposure on behavioral outcomes in rodents is summarized. Using meta-analyses, all the available data was analyzed. **Chapters 3-6** describe early life stress studies with rats. In **Chapter 3**, $SERT^{+/+}$, $SERT^{+/-}$, and $SERT^{-/-}$ males and females were exposed to early life stress to assess the impact of genotype and stress on the gut microbiome. In **Chapters 4-6**, only $SERT^{+/-}$ female rats were used. These females possess a vulnerable genotype, and because we use them to model aspects of maternal mood disorders, we call them maternal vulnerability (MV) females. MV females exposed to stress early in life (sMV) show anhedonia in adulthood, while MV females exposed to control handling (cMV) do not. sMV and cMV females were treated with the SSRI fluoxetine (FLX) or vehicle (Veh) from the start of gestation until the end of lactation. Biological outcomes were assessed in sMV and cMV females (**Chapter 4**), fetal tissue (**Chapter 5**), and offspring (**Chapter 6**).

Overview of findings

Offspring behavior

The systematic review in **Chapter 2** combines data from 99 previously published studies on behavioral outcomes in rodents after perinatal SSRI exposure. We performed 9 separate meta-

analyses corresponding to different behavioral domains. Using data from thousands of animals, we found that perinatal SSRI exposure increases activity, leads to a more passive stress-coping style, and less efficient sensory processing. We found a non-significant trend towards more anxiety in adulthood after perinatal SSRI exposure. Females are understudied in this field, but the available data indicates that male offspring may be more vulnerable to the effects of perinatal SSRI exposure.

The maternal gut microbiome

In **Chapters 3 and 4**, we studied the gut microbiome in relation to SERT genotype, early life stress and/or SSRI administration. **Chapter 3** describes a study comparing the gut microbiome profiles of $SERT^{-/-}$, $SERT^{+/-}$, and $SERT^{+/+}$ juvenile animals with or without exposure to early life stress. We found that SERT genotype was associated with characteristics of the gut microbiome. The composition of the gut microbiome in $SERT^{-/-}$ juvenile rats is distinct from that of their $SERT^{+/-}$ and $SERT^{+/+}$ counterparts. Meanwhile, early life stress is also linked to changes in the gut microbiome. Specifically, it is associated with a decrease in *Bacteroidetes* and an increase in *Firmicutes*. Animals with low SERT expression plus exposure to early life stress showed a flourishing of microbes usually linked to inflammation. In **Chapter 4**, the gut microbiome of previously stressed and control handled $SERT^{+/-}$ (sMV and cMV) females was investigated during pregnancy and lactation. Apart from a clear distinction between the microbiome during pregnancy vs lactation, we found that FLX impacted the maternal microbiome most clearly in the sMV females. In particular, sMV-FLX females had a lower relative abundance of *Bacteroides*, and a higher relative abundance of *Prevotella* and *Ruminococcus* than sMV-Veh females. Additionally, sMV-FLX females showed lower fecal concentrations of the amino acid serine during pregnancy, and aspartic acid during lactation than sMV-Veh females. This might reflect decreased transfer of amino acids to the developing offspring.

Offspring placental and brain gene expression

In **Chapters 5 and 6**, we investigated potential molecular mechanisms involved in the long-term effects of exposure to a mother with a depressive-like phenotype and/or SSRI antidepressants during the perinatal period. In the placenta, we did not find major differences in gene expression related to the stress- and serotonin system, neurogenesis, signal transduction, or angiogenesis as a result of exposure to the sMV phenotype and/or SSRI treatment (**Chapter 5**). This was unexpected, since the genes we tested were previously shown to be altered as a consequence of maternal depression and SSRI treatment in humans. In juvenile offspring, however, we identified brain region- and sex-specific effects on myelin-related gene expression as a result of exposure to the sMV maternal phenotype and FLX (**Chapter 6**). Specifically, males showed larger differences in myelin-related gene expression than females. In addition, we identified a potential role for epigenetic regulation in these effects, as gene expression of myelin-associated glycoprotein (*Mag*) and Myelin basic protein (*Mbp*) correlated negatively to DNA methylation around the promoter sites of these genes.

The mechanisms behind the long-term neurobiological effects of exposure to maternal depressive-like symptoms, SSRIs, or both

Behavioral outcomes

Stress exposure during early development

Rodent models of maternal depressive-like symptoms usually involve applying a stressor to the dam and/or pups, either prior to pregnancy, during pregnancy, or during the early postnatal period. Our own studies using pre-gestational stress have shown that a maternal sMV phenotype is associated with several sex-specific behavioral outcomes in offspring[12], for example in the social[13] and emotional[14] domains. A study from a different lab examining timing- and sex-specific effects of *in utero* stress exposure found that especially early prenatal stress had effects on adult offspring stress responsivity and depressive-like behaviors, and that mainly male offspring were vulnerable to this[15]. Other behavioral domains that have been shown to be affected by gestational stress in animal models are learning and memory, general locomotor behavior and exploration, and emotional behaviors such as anxiety and anhedonia[16,17]. The effects of early *post*natal stress cover similar behavioral domains, and also here, males tend to be more likely to develop long-term effects[18].

SSRI exposure during early development

We summarized and analyzed all available evidence of behavioral outcomes after perinatal SSRI exposure in laboratory rodents in **Chapter 2**. Combining data of 99 publications covering a wide range of behavioral tests, we found evidence for significantly heightened activity scores, a more passive stress coping style, and less efficient sensory processing in adult animals after early exposure to SSRIs. Although females are still understudied in this field, subgroup analyses indicated that they usually show weaker behavioral effects than males. We examined timing of exposure (prenatal, postnatal, or both) as a potential moderator of the effects, and found that early *post*natal exposure was associated with the strongest effects.

Recent behavioral studies from our own lab[12] were not included in these meta-analyses because they were not published on or before the date of the literature search. However, results indicate that juvenile social play behavior is reduced in both males and females after perinatal SSRI exposure, while social behavior in adulthood is only reduced in males[13]. In contrast, anxiety and anhedonia were increased in female, but not male, FLX-exposed offspring[14]. Some social behaviors were only assessed in males; FLX-exposed males show lower aggression than controls, while overall sexual performance is unaffected[19].

Do developmental stress and SSRI exposure interact?

In general, perinatal FLX exposure has stronger effects on behavior than the maternal sMV phenotype in our experiments[12]. However, the two interact to affect the outcome of some behavioral tests. For example, social play behavior was reduced in FLX-treated female offspring from cMV

mothers, but not from sMV mothers[13]. The same was true for total social behavior of males in adulthood[13]. Other labs have found similar results, where perinatal SSRI treatment normalized the inhibiting effect of maternal pre-gestational stress on rat juvenile play behavior in both sexes[20]. A study in rats found that immobility time in the forced swim test was *decreased* in adulthood after exposure to prenatal stress[21], while another study in mice found that it was *increased*[22]. In both studies, *in utero* exposure to SSRIs prevented the effects on immobility[21,22]. However, findings on interactions between prenatal stress and SSRI exposure are not always replicable[23]. Moreover, our meta-analyses in **Chapter 2** suggest that, despite individual study outcomes, the overall behavioral effects of perinatal SSRI exposure do not depend on the presence or absence of maternal stress.

The neurodevelopmental basis

Myelin-related gene expression is affected in a sex- and region-specific manner

To better understand the neurobiological basis of these behavioral alterations, we investigated whole-genome transcriptomics in the brain of juvenile rats in **Chapter 6**. This hypothesis-generating approach indicated that myelin-related genes were changed in expression between offspring exposed to the maternal sMV phenotype versus controls, and in offspring exposed to SSRIs during early development versus controls. Myelination is a crucial process for brain health, as multiple lipid layers wrap around axons to facilitate faster nerve conduction[24]. During neurodevelopment, myelination is involved in neuronal circuit formation[24]. We found that juveniles exposed to the maternal sMV phenotype, as well as animals exposed to FLX, showed an upregulation of myelin-related genes in the prefrontal cortex (PFC), but a downregulation of these genes in the basolateral amygdala (BLA) compared to controls. This was especially true in males – females only showed this same pattern in the BLA. At the level of individual genes, those coding for myelin–associated glycoprotein (*Mag*), myelin basic protein (*Mbp*), claudin-11 (*Cldn11*) and 2',3'-cyclic-nucleotide 3'-phosphodiesterase (*Cnp*) showed an interaction between sMV and FLX exposure. The group exposed to both interventions resembled the control group, suggesting that FLX might have normalizing effects on the expression of these genes in sMV-exposed offspring.

Is altered myelination an explanation for behavioral outcomes?

These transcriptomic data from **Chapter 6** share several key features with the behavioral data from the literature on perinatal stress exposure and the findings described in **Chapter 2**. First, the effects of maternal stress and exposure to SSRIs during development are strikingly similar. In behavior, both maternal stress and perinatal SSRI exposure have been reported to be associated with changes in activity and stress coping. In our neurobiological data, both sMV and FLX are associated with changes in myelin-related gene expression. Second, males show stronger effects of exposure to sMV and FLX both on a behavioral and a transcriptomic level. Third, on a behavioral level, early *post*natal SSRI exposure leads to the strongest effects. This is congruent with the timing of myelination which, in rodents, begins in the early postnatal period[25]. Fourth, similarly to interaction effects found in some behavioral and neurochemistry studies of maternal stress and SSRI administration[21,22,26], sMV and FLX interact to influence gene expression levels of myelin-related genes *Mag*, *Mbp*, *Cldn11*, and

Cnp. For these reasons, it is plausible that altered myelination is part of the biological cascade linking exposure to maternal depressive-like symptoms and SSRIs during early development to long-term behavioral outcomes.

Does altered myelination reflect altered brain maturation?

Two interesting features of the effects that we found on myelin-related genes in **Chapter 6** are: 1) highly dependent on brain region, but 2) not dependent on the nature of the intervention (sMV or FLX). For these reasons, we speculate that these findings reflect a difference in general brain maturation. At three weeks of age, myelination in rats is already spread throughout the brain, with the corticolimbic system among the latest-myelinating regions[25]. We propose that the corticolimbic system myelinates earlier following exposure to stress or SSRIs, with the peak of BLA myelination accelerated to before PND21 (and thus leading to a *decrease* in myelination at this time point), and PFC myelination accelerated to around PND21 (leading to an *increase* in myelination at this time point). This has yet to be confirmed experimentally, but is congruent with other reports linking early stress[27,28] and SSRI exposure[29] to accelerated or abnormal brain maturation. It is also congruent with other types of interventions in the perinatal period leading to changes in myelination and behavioral outcomes, such as was shown for protein deficiency[30], and inflammation[31], suggesting that these effects are fairly unspecific but all involve altered brain maturation.

The role of epigenetic regulation

Environmental influences during the perinatal period that affect (neurobiological) outcomes are often thought to act through epigenetic processes[32]. Early stress exposure has been associated with epigenetic regulation of stress-related genes with long-term effects[33]. Early SSRI exposure has been associated with changes in proteins responsible for histone modifications and DNA methylation, as well as epigenetic regulation of genes such as brain-derived neurotrophic factor (*Bdnf*)[34]. In **Chapter 6**, we measured DNA methylation around the promoter regions of *Mag*, *Mbp*, *Cldn11* and *Cnp*. The DNA was isolated from the same tissue punch as the RNA that was used for transcriptomics, allowing for direct correlations between gene expression and DNA methylation levels. *Mag* and *Mbp* gene expression was negatively correlated to their levels of DNA methylation. This is in line with the notion that DNA methylation at the promoter region usually silences the gene. Our data highlights the possibility that sMV- and FLX-induced changes in myelination are facilitated by an epigenetic mechanism.

The maternal gut microbiome

Serotonin (transporter) and the gut microbiome

In recent years, the gut-microbiome-brain axis received increased attention. It has become clear that the microbial community inhabiting the gastrointestinal system communicates with the nervous system in a bi-directional manner[35]. (Early life) stress and serotonin regulation are two important factors of interest in this field. In humans, major depressive disorder has been associated with specific gut microbiota signatures[36–42] and rodents with depressive-like symptoms show altered gut microbiota composition[43–47]. Interestingly, microbes synthesize tryptophan, the precursor to

serotonin, and stimulate serotonin biosynthesis by the host[48]. Vice versa, host serotonin regulation affects the growth of some microbes[49].

However, it was not yet clear how host SERT genotype would affect the composition of the gut microbiome and if it shows any interactions with early life stress. In **Chapter 3**, we investigated this in juvenile male and female rats. Our results showed that animals with SERT knocked out had different gut microbial communities than their wildtype counterparts, with heterozygous animals showing an intermediate phenotype. Rats with a (partial) knockout of SERT that were exposed to early life stress, showed features of the microbiome that are associated with inflammation. There were no sex differences. Our data suggests that lowered SERT activity, in particular when combined with early life stress, is associated with dysbiosis of the gut microbiome in young animals, with potential consequences extending into adulthood.

The microbiome during pregnancy and lactation

Because our sMV females were heterozygous for SERT and exposed to stress early in life, the natural next question was whether these changes in the microbiome would persist through pregnancy and lactation. Since SSRIs target the SERT, we were also interested in whether the maternal microbiome may be altered by antidepressants. In fact, during the writing of this PhD thesis, the effects of psychoactive drugs such as antipsychotics, antidepressants, and mood stabilizers on the gut microbiome have been the focus of many new studies[50]. Recent studies of the gut microbiome in humans[42,51,52] and rodents[53–58] revealed that SSRI antidepressants alter the composition of the gut microbiota *in vivo*. This may be related to these drugs' antimicrobial activity as was shown *in vitro*[53,59,60].

In **Chapter 4**, we examined the effects of a depressive-like phenotype and SSRI administration in females on features of the gut microbiome in the perinatal period. The maternal gut microbiome during pregnancy and early postpartum had never been directly compared before. We showed that the microbiome during pregnancy had a higher alpha diversity than during lactation, and its composition also changed dramatically. We examined the most prominent changes in the microbiome between pregnancy and lactation, and showed that FLX treatment affected the relative abundance of certain microbes most clearly in sMV females. The results from **Chapter 3** and **Chapter 4** together suggest that the combination of $SERT^{+/-}$ genotype, early life stress, and FLX treatment is associated with gut microbiome characteristics that are most different from the control.

It is known that microbes in the gut produce metabolites that are used by the host[61–63], and that microbial products during pregnancy can affect offspring outcomes[64,65]. We examined the metabolite profile of maternal feces during the perinatal period (**Chapter 4**) by performing targeted metabolomic analyses to measure amino acids, short-chain fatty acids, and bile acids. Just as the microbiome, the metabolite profile during pregnancy is distinct from the profile during lactation. FLX treatment impacted metabolite availability most strongly in feces of sMV females. Amino acids such as serine and aspartic acid were reduced most strongly in sMV-FLX females, with potential consequences for their offspring.

Is the maternal microbiome related to (neuro)behavioral outcomes in offspring?

Microbial molecules have been shown to transfer from mother to offspring during gestation and through lactation to support the development of the brain and the immune system, emphasizing their importance for offspring development[64–67]. Of interest in the context of this thesis, the relationship between the gut microbiome and behavior is mediated by central nervous system myelination in some instances[68]. For example, germ-free mice show an upregulation of myelin-related genes in the prefrontal cortex compared to their normally colonized counterparts[69]. Another study showed that after a fecal transplant containing specific microbes, prefrontal cortex myelination and social behavior were altered in mice[70]. Whether the alterations in myelin-related gene expression (**Chapter 5**) and behavior[12] that we observed in our animals are mediated by the microbiome remains to be established. Moreover, none of the available studies on the microbiome and myelination focuses on the *maternal* microbiome. Future research may shed light on whether the changes described in the maternal gut microbiome and its metabolic output by FLX mediate its effects on offspring myelination during neurodevelopment. However, alternative pathways affecting offspring behavior should be explored as well.

The placenta

The placenta is a specialized organ allowing for transfer of glucose and nutrients from the maternal to the fetal blood circulation during pregnancy[71]. There is evidence that the placenta is an important mediator of the effects of gestational maternal stress on offspring neurodevelopment[72]. Both increases and decreases in serotonin levels may affect placental functioning[73]. In fact, it has been suggested that the placenta may play a role in the long-term effects of maternal depressive-like symptoms and SSRI use during pregnancy on offspring health outcomes[74]. Human studies of placentas sourced from pregnancies characterized by depression and antidepressant use have highlighted stress- and serotonin-related, neurogenesis-related, and angiogenesis-related gene alterations. These studies typically do not allow to easily disentangle the effects of depression from the effects of SSRIs.

In order to examine the separate and combined effects of a maternal depressive-like phenotype and SSRI exposure, we aimed to reproduce these effects in a rat experiment (**Chapter 5**). We measured the expression of specific stress- and serotonin-related (*Nr3c1, Maoa*, and *Slc6a4*), neurogenesis-related (*Bdnf*, *Igf1r, Ngf*, and *Npy*), signal transduction-related (*Mapk1* and *Mapk3*) and angiogenesis-related (*Pgf*, *Rock1*, and *Rock2*) genes in near-term male and female rat placentas. We found that a maternal sMV phenotype was associated with lower *Mapk1* expression. However, we did not identify other significant differences in placental gene expression as a result of maternal adversity or SSRI administration. There are several potential explanations for this. For example, we only examined the fetal part of the placenta, whereas many human studies do not use a specific region. Further, we examined the expression of 12 genes but there are conceivably other targets that might have been differentially expressed. However, subgroup analysis of behavioral data suggested that especially early *post*natal SSRI exposure has an effect on offspring outcomes. This indicates that the role of the placenta in mediating these effects, at least in rodents, may be limited.

Reflections on the animal model

Interaction between the SERT genotype and stress in humans

The initial study by Caspi and colleagues, showing that the likelihood of developing depression after experiencing stressful life events is influenced by SERT genotype[75], gained a lot of attention and was followed by many replication studies and heated scientific debate that continues to this day. By 2009, a sufficient number of studies had been published to allow for meta-analyses of aggregated data. The first systematic reviews concluded that there was no increased vulnerability of "s" allele carriers on developing depression after stressful events[76,77]. These studies were criticized for using stringent inclusion criteria, including only 5[76] and 14[77] studies[78]. For example, studies examining childhood maltreatment were excluded, even though this was examined in the original Caspi study[78]. A follow-up meta-analysis, including all 54 available unique datasets, reported strong evidence for a modulatory role of the SERT polymorphism in the relationship between stress and depression[78]. This was later confirmed in an updated meta-analysis including 27 additional studies[79]. Whether an overall interaction effect is found or not clearly depends on which studies are included in the analysis.

In 2013, a novel protocol was published for a large-scale collaborative meta-analysis[80]. To reduce bias as much as possible, it proposed to re-analyze the raw data using pre-defined methods[80]. Illustrating the controversial nature of the topic, this protocol was criticized by the authors of the original study for excluding publications with fewer than 300 participants, and for including lifetime depression as an outcome measure[81]. The final published meta-analysis did not support the hypothesis that the interaction between the SERT genotype and stress exposure changed the risk of developing depression[82]. Rather, it reported that life stressors constitute a strong risk factor, while SERT genotype does not[82]. The controversy continues as researchers become increasingly interested in the sources of variation between studies. These sources of variation, e.g., the presence of subtypes of depression[83] and differences in the assessment of stress and depression,[84] might explain the discrepancies in reported research results. Currently new, extremely large datasets are being generated with hopes of finding answers to these questions[85]. However, it is clear that the proposed interaction between stressful (early) life events and SERT genotype in the development of depression, if even real, is not as straightforward as it might have seemed in the past.

Interaction between the SERT genotype and stress in laboratory rodents

Overview of the literature

To further investigate this gene X environment interaction, researchers have studied the differential effects of stress exposure during adulthood on wildtype versus $SERT^{+/-}$ rodents[86], which have SERT transcription reduced by 40-50% (this is comparable to human "s" allele carriers)[87–89]. In one study, researchers exposed adult male $SERT^{+/+}$, $SERT^{+/-}$, and $SERT^{-/-}$ mice to either a winner or loser experience in confrontation with another male[90]. Within the loser group, homozygous knockout males displayed more anxiety-like behavior than males with other genotypes[90]. This is supportive of the presence of a gene X environment interaction, but only in an "extreme" genotype that does not

occur in humans. Another study, arguably more translationally relevant, subjected adult male wildtype and heterozygous SERT knockout mice to chronic stress for three weeks[91]. Only the SERT$^{+/-}$ males were sensitive to the stressor as indicated by altered locomotor activity and social avoidance, suggesting these outcomes show a SERT X stress interaction[91].

Stress during adolescence has also been studied in this context, for example with the repeated shock exposure paradigm[92]. Stress-exposed SERT$^{+/-}$ animals showed a higher freezing response to the shock context than non-exposed SERT$^{+/-}$ animals. In contrast, wildtype animals showed no difference between exposed- and non-exposed animals[92], suggesting the presence of a SERT X stress interaction. However, another study investigating the effects of an adverse social situation during adolescence reported no interaction effects on anxiety or aggressive behavior[93]. Overall, it should be noted that although some SERT X (adult or adolescent) stress interactions were found in the studies mentioned above, many outcomes were affected by only SERT genotype and/or only stress exposure.

Most studies in laboratory rodents have investigated *early life* stress exposure, analogous to the findings that childhood maltreatment increased the likelihood of developing depression stronger in "s" allele carriers from the initial Caspi study[75]. Although there is evidence for altered HPA axis response to acute stress in SERT$^{+/-}$ rodents compared to their wildtype counterparts, there is limited evidence for altered (physiological or behavioral) stress coping after early life stress exposure depending on SERT genotype[89]. There are reports of SERT$^{+/-}$ mice and rats being more vulnerable to disruptions in early maternal care in developing anxiety- and depressive-like behaviors during adulthood[94–96]. However, several studies showed no modulating effect of SERT genotype[97,98]. In that sense, the animal work seems to correspond to the conflicting results from human cohorts[82]. It has been suggested, however, that the stressors used in animal studies might not have been severe enough to induce long-term effects[89]. It is important to note that researchers studied almost exclusively male rodents, whereas mood disorders are in fact more prevalent in females[89], and evidence from rhesus macaques suggests that female "s" carriers may be more sensitive to the effects of early life stress than males[99].

Our maternal vulnerability model

The work described in this thesis is based on a rat model of maternal vulnerability (MV), referring to SERT$^{+/-}$ dams that have either been exposed to stress early in life (sMV) or to control handling (cMV). The early life stress procedure was based on daily maternal separation from PND2 until PND15. Addressing concerns that previous studies might have not used stressors sufficiently severe to induce depressive-like behavior in SERT$^{+/-}$ animals[89], we opted to administer 6 hours of daily maternal separation instead of the typically seen 3 hours[100].

Two studies, described in detail elsewhere[12], were performed to assess whether this procedure indeed induces depressive-like symptoms in MV females. The first study compared sMV and cMV females in adulthood, but pre-gestation[101]. Behaviorally, sMV females showed reduced sucrose preference over cMV females. Neurologically, sMV females had lower gene expression of nerve growth factor in several brain regions. We concluded that sMV females displayed anhedonia, a key endophenotype related to depressive-like symptoms[101]. However, no differences were found

in anxiety (as assessed using the open field test and elevated plus maze), sociability and social recognition, stress coping, and basal corticosterone levels. This suggests that the depressive-like phenotype of sMV females is mild.

A second study was performed to investigate SERT genotype X early life stress interactions in this model[12], as well as the effectiveness of our current maternal separation protocol. To this end, $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ pups were subjected to one of several maternal separation variations: either control handling, 6-hour maternal separation a day at a predictable time (our current protocol), 3-hour maternal separation a day at a predictable time, 3-hour maternal separation a day at an unpredictable time, or 3-hour maternal separation a day at an unpredictable time with additional stress exposure for the mother. In adulthood, females from these treatment groups were tested for anxiety-like behavior, memory performance, and depressive-like behavior. Aside from main effects of genotype and early life stress exposure, the only interactions between these two on adult behavior were driven by the $SERT^{-/-}$ females. Further, the difference in sucrose preference between sMV and cMV females, as described above, was not replicated in this second study. This sheds more doubt on the depressive-like nature of sMV females.

The current thesis does describe some outcome measures suggesting that sMV females are phenotypically distinct from their cMV counterparts. The gut microbiome composition differs, both at 3 weeks of age (**Chapter 3**) and in adulthood (**Chapter 4**). In addition, offspring gene expression profiles from sMV dams are distinct from offspring of cMV dams (**Chapter 6**). Earlier work has also described (limited) behavioral differences between offspring of sMV versus cMV mothers[12]. It is possible that the nature of the phenotypic difference between sMV and cMV females was not captured in the (mainly behavioral) tests. An important caveat to all tests that were performed on sMV versus cMV females is that they did not take place during the perinatal period (except for microbiome analyses in **Chapter 4**), even though we used the sMV females as a model for *perinatal* depression. Interestingly, it has been reported that stress-related symptoms are often uncovered during hormonally dynamic periods such as pregnancy, highlighting the possibility that a depressive-like phenotype could be present in sMV females specifically in this period[102]. Unfortunately, this has not been investigated.

SSRI treatment in $SERT^{+/-}$ rats

Do SSRIs work differently in humans depending on SERT genotype?

The experimental work in this thesis is based on SSRI treatment – blocking SERT – in rats that are already genetically SERT deficient. These rats mimic a relatively common genetic variant in humans, the "s" allele of the serotonin transporter gene promoter polymorphism (5-HTTLPR). Since "s" allele carriers in the human population are also commonly prescribed SSRIs, researchers have wondered if their response to this class of drugs is different from those with higher levels of SERT. This has clinical relevance in that it could help personalize treatment based on genotype[103].

The first meta-analysis addressing this question concluded that SERT genotype was significantly associated with SSRI treatment outcome; patients homozygous for the "s" allele had lower response- and lower remission rates after 4 weeks of treatment than those with at least one "l"

allele[104]. However, a more recent meta-analysis concluded that there was no significant effect of SERT genotype on SSRI response, and only a weak, if any, effect on remission[105]. Apart from the effectiveness of the drug, its potential side effects have also been monitored in the context of SERT genotype. A review of the literature concluded that "s" allele carriers have a greater adverse event load[106]. Side effects such as mania, insomnia, agitation, fatigue, sweating, and dizziness have been reported more commonly in "s" allele carriers[106]. However, there is no evidence that carrying the "s" allele increases the likelihood of discontinuation from SSRI treatment[107], suggesting that, in general, any side effects are perceived as tolerable by "s" allele carriers.

Unexpected toxicity

We treated sMV and cMV females daily with 10 mg/kg FLX or Veh throughout pregnancy and lactation. Females were treated orally, by using flexible feeding tubes and without restraint, to minimize stress and discomfort. However, we observed more attempts to avoid the treatment in some FLX-treated females after repeated daily treatment. Unexpectedly, we witnessed elevated mortality rates in the experimental group: about 25-30% of FLX females died during the experiments compared to none in the Veh-treated group. There was no discernable pattern in the timing of death. Females died during pregnancy as well as postpartum, and within minutes to hours after the last FLX injection. There was also no difference between sMV and cMV females. Unaffected females did not show overt symptoms of toxicity, except for lower weight gain and sporadically some piloerection. Effects of FLX on food intake and weight gain during the perinatal period are normal and have been observed previously in rats after a 12.5 mg/kg[108] and 17 mg/kg[109] daily dose, but mortality was not reported in these studies. In fact, other rodent studies of perinatal SSRI treatment have used doses up to 30 mg/kg/day without lethal outcomes (**Chapter 2**).

Since none of the studies from other labs were performed in $SERT^{+/-}$ females, it is possible that the observed toxicity is related to genotype. The serotonin syndrome is a collection of symptoms caused by excessive serotonin levels in the body. It is usually induced by the combined use of two serotonergic drugs with different mechanisms of action, although it has been described in individuals only taking one drug[110]. Lethal toxicity in humans has only been reported after use of an SSRI in combination with a different serotonergic drug[111]. $SERT^{+/-}$ mice have been proposed as a rodent model relevant to the serotonin syndrome[112], as they show some hypersensitivity to SSRIs[113,114] and increased drug-induced responses that resemble the human serotonin syndrome responses[115].

Evidence from human and animal work suggests that serotonergic activity is elevated during normal pregnancy[116,117]. However, higher than average serotonin levels during pregnancy have been consistently associated with preeclampsia, a serious disease characterized by high blood pressure[117]. In fact, serum levels of serotonin correlate with the severity of preeclampsia symptoms[118]. Interestingly, preeclampsia has overlapping symptoms with obstetric serotonin syndrome[119,120]. Prenatal SSRI use is also known to increase the risk of developing gestational hypertension and preeclampsia[121], providing further evidence of the link between high serotonin levels during pregnancy and the risk for adverse maternal outcomes. Under control conditions, there is no difference in blood pressure between $SERT^{-/-}$, $SERT^{+/-}$, and $SERT^{-/-}$ females[122]. However, this

might be different during pregnancy. Overall, surprisingly little is known about the serotonin system and mechanisms of action of SSRIs during pregnancy[116].

Drug metabolism might also be altered during pregnancy. A recent study comparing pregnant to non-pregnant rats that were treated with the SSRI sertraline showed that serum levels of the drug and its metabolite norsertraline were significantly higher in case of pregnancy[123]. One potential explanation the authors offer is that key enzymes involved in drug metabolism, cytochrome P450 enzymes, are reduced during pregnancy, and thereby could affect drug levels[124]. Our own data indicated that there was no accumulation of fluoxetine or norfluoxetine in blood plasma over the course of treatment[12]. However, we did not compare the levels to those in non-pregnant animals and we measured levels of the drug 23 hours after the last injection. It has been suggested that bolus daily injections may lead to transient high serum concentrations of the drug[125].

Taken together, it seems possible that the observed mortality was caused by a combination of the SERT$^{+/-}$ genotype, bolus injections of SSRIs, and changes in serotonin levels and/or drug metabolism associated with pregnancy. However, in pilot studies from our own lab where SERT$^{+/-}$ dams were treated with fluoxetine, we did not observe any mortality. It is also puzzling that most SERT$^{+/-}$ females were unaffected by FLX treatment. The obvious experiment to perform would be to directly compare SERT$^{+/-}$ dams to wildtypes. At the time of writing, such a study has not been conducted.

Implications and recommendations

Rodent research: future perspectives

As is the case with most research, the studies described in this thesis generate more questions than they answer. Below is a short overview of potential directions for new studies building on the current findings.

Maternal vulnerability model

The depressive-like symptoms of the sMV females seem to be mild. There are three directions of research that could increase the translational value of the MV model: optimizing the stressor (increasing construct validity), expanding the test battery and selecting sensitive animals (assessing and increasing face validity), and testing the response to antidepressants (assessing predictive validity). Regarding the stressor, only stress early in life has so far been applied to MV females. However, even in the original Caspi study, it was proposed that the "s" allele increased the probability of a depressive episode as a factor of the *number* of stressful life events[75]. It might be worthwhile to explore whether multiple stressors, spread over multiple phases of life, would result in a stronger depressive-like phenotype in MV females. Regarding the test battery, there are no studies that examine the depressive-like phenotype of sMV females specifically in the perinatal period. This despite the fact that we use it as a model for perinatal depressive-like symptoms, and the potential for this hormonally dynamic period to unmask the effects of early life stress. Therefore, it would be of interest to look at stress-coping behavior, anxiety-like behavior, and anhedonia during pregnancy or postpartum. In addition, these behaviors could be assessed pre-conception, to allow

for the selection of only those animals that show the desired behaviors. Regarding the response to antidepressants, running this test battery before and after treatment would help discern whether the animals show the expected response to the drug.

It is important to realize that we can only model *aspects* of maternal depression in rodents, and not the entire condition[126]. For one, depression in humans has a large psychological component that is difficult to observe, if not simply absent, in rodents[127]. Secondly, depression is a highly heterogeneous disorder, with various etiologies and diverging symptoms[126]. For the benefit of those involved in animal research of psychiatric disorders, as well as outsiders, it might be helpful to clarify and put more emphasis on the *aspect* of the psychiatric disorder that the animal model is supposed to represent. The strongest feature of our MV model of $SERT^{+/-}$ females exposed to early life stress is its construct validity. However, its face validity and predictive validity are largely unclear.

Perinatal FLX treatment in MV females

The fact that some MV females died after FLX treatment is worrying both from an ethical- and scientific standpoint. It is important to establish why this happened. First, FLX could be administered to both $SERT^{+/-}$ and $SERT^{+/+}$ females, pre-gestation and during the perinatal period, to determine whether this effect is dependent on genotype and/or reproductive stage. Females could be monitored 24/7 (e.g., in PhenoTyper cages) to track potential serotonin syndrome-related symptoms. Additionally, plasma levels of FLX and serotonin could be monitored more frequently throughout the treatment period, and during different times of the day. Finally, alternative methods of drug administration that are less likely to result in heightened drug concentration[125], such as administration in biscuits several times a day[20], should be considered.

This unexpected toxicity of FLX raises concerns about the translational relevance of the studies. Even though the surviving females did not show signs of toxicity, there is a possibility that they were affected by FLX in ways that are quantitatively or qualitatively different than is usually the case in humans. One outstanding question is whether the maternal care provided to the offspring was altered by FLX treatment. Systematic observations of maternal care provided by MV females could shed light on the potential modulating role of maternal care in the neurobiological and behavioral effects of the maternal depressive-like phenotype and FLX treatment on offspring. Until the cause of the observed mortality is clarified, wildtype females – exposed and unexposed to (early life) stress in combination with perinatal SSRI treatment – should be preferentially considered for future studies.

Offspring behavior

Following from the synthesis of evidence in **Chapter 2**, some areas of future research on the behavioral effects of perinatal SSRI exposure deserve particular attention. First, all future studies should include both males and females, to account for the likely significant sex-specific effects. A better understanding of sex-specificity is translationally relevant and might also inform mechanistic studies. Second, more studies combining models of maternal depressive-like symptoms with SSRI treatment (see above) are needed to further study their potential interactions. As the number of such studies increases over time, a separate meta-analysis including *only* these studies would be valuable.

Third, it would be of interest to explore the behavioral category of anxiety: in our preliminary analysis it appeared the elevated plus maze test did not reveal a difference between SSRI-exposed and unexposed offspring, while other tests of anxiety do. If this holds true in future explorations, could it be that these tests measure different constructs? And what does that mean for the interpretation of the results? Fourth, to further clarify the distinct effects of exposure to SSRIs at different points in development, future meta-analyses could include data from SERT knockout animals, analogous to an extreme form of chronic, life-long SSRI treatment. Lastly, we hope that the publication of our systematic review will motivate researchers in this field to be more diligent in their study design and reporting of outcomes. We hope that our systematic review and meta-analysis will be updated in the future to include the most recent studies as they become available.

Offspring neurobiology

In **Chapter 5**, we described alterations in the expression of myelin-related genes, dependent on brain region and sex, as a result of perinatal exposure to a maternal depressive-like phenotype and SSRI medication. There are many questions that follow from these results. First, our results are based on a limited number of animals, which creates a strong need for replication studies. Further, it is necessary to validate these results on the protein level: are altered myelin-related transcripts actually reflected in altered protein levels? Integrated transcriptomic- and proteomic analyses would be valuable here, and analyses of actual myelination levels and the structure of the white matter could be investigated using imaging techniques. Whole-brain analyses such as these would also shed light on how various brain structures outside of the PFC and BLA are affected, and how circuit formation might be affected by early exposure to maternal stress and/or SSRIs. Lastly, we only performed transcriptomics on PND21 brains. A time-course experiment to gather more information about how the developmental trajectory might be altered may be of value.

Maternal microbiome

In **Chapters 3 and 4**, we describe the effects of early life stress and serotonin transporter functioning on the gut microbiome during several periods of life. The interactions between serotonin regulation and the microbiome is a rapidly developing field. More studies are needed, particularly considering that replication of results has proven difficult (see Discussion **Chapter 4**). Studies of the gut microbiome during pregnancy and lactation are sparse and necessary to further characterize the typical changes in microbiome composition and output that occur during this dynamic period of life. Of particular interest will be further explorations of the relationship between the maternal gut microbiome and the availability of metabolites. We found low concentrations of some amino acids in the feces of sMV-FLX females (**Chapter 4**), but it is unclear whether this is related to low systemic availability, and decreased transfer to the offspring. Maternal blood and offspring blood as well as brains could be analyzed for metabolomic alterations as a result of FLX treatment.

Placenta

We did not identify major changes in gene expression using targeted qPCR assays in placentas from pregnancies of depressive-like and/or FLX-treated rats (**Chapter 6**). This does not mean that there

is truly no difference in placental functioning – our sample size was limited. This also precluded sex-specific analyses, despite knowing that on a behavioral- and neurobiological level there are sex-specific effects, and the placenta has been previously implicated in sex-specific effects of prenatal insults[128]. Whole-genome transcriptomics combined with proteomics on placental samples could be performed to fully capture any differences that might occur. In addition, a longitudinal approach could be used to track changes in the placenta over the course of pregnancy, instead of only examining them at term.

Human research: future perspectives

Human term placentas too can be examined relatively easily, although also here, a whole-genome transcriptomics approach has not been extensively used yet. Similarly, microbiome studies are practically feasible in humans. Longitudinal tracking of the fecal microbiome over pregnancy and lactation could be performed, with pregnancies characterized by maternal mood disorders and SSRI treatment being of particular interest. Maternal and offspring blood could be used for metabolite profiling. Longitudinal tracking of white matter development throughout infancy and childhood after exposure to maternal depression and SSRI treatment could be used to investigate potential differences in brain development trajectory. In general, behavioral and neurobiological studies of exposed offspring extending into adolescence and adulthood are very rare, and would likely provide valuable insights.

Conclusion

The aim of this PhD project was to use a rat model of maternal depressive-like symptoms to examine potential mechanisms linking perinatal SSRI antidepressant exposure to long-term health outcomes. We identified changes in the maternal microbiome and metabolic output, and changes in the transcriptomic state in the developing offspring brain that could be mediating the long-term effects of exposure to maternal depression and antidepressant use during pregnancy. The results can be used to inform the design of future animal- and human research in this field; sex-specific effects, white matter development and brain maturation, and the role of the maternal microbiome emerge as the most relevant areas of focus for future study. Our hope is that by informing animal- and human research in this field, the current thesis will help develop a better foundation to the knowledge that lies at the core of clinical decisions around perinatal mood disorders, and help mitigate the potential adverse effects of treatment (or lack thereof) on both mothers and children.

This thesis is part of a larger field of study that focuses on early development as a period of particular sensitivity to environmental influences that affect long-term health outcomes and happiness. Effective management of the prevalent stress and mental health issues is of particular relevance during the perinatal period. Indeed, depression does not only negatively affect the quality of life of the affected woman, but has the potential to result in severe intergenerational consequences. As scientists continue to conduct human and animal experiments with the aim to unravel the potential mechanisms by which depression and antidepressant use affect children, evidence-based

decision making focused on the long-term health and wellbeing of individuals, as well as society as a whole, will become increasingly possible and should become priority.

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