

The Serotonin Transporter and Early Life Stress: Do they interact and induce an anxiety-like phenotype?

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Abstract

Variation of the serotonin transporter (SERT) gene was suggested to exert a modulating effect on the association between early life stress (ELS) and the risk for anxiety and/or depressive disorders. Rodents with diminished SERT function seem more anxious than wildtypes. ELS, induced by low maternal care, is known to increase anxiety like behaviour in rats in general. In addition, genotype x environment (GxE) interactions are proposed to play a key role in behavioural alterations found in animals with vulnerable genes who were exposed to adverse environments. In the present study we investigated whether ELS in female rats with different SERT genotypes would alter affective behaviour. To this extent, SERT^{+/+}, SERT^{+/-} and SERT^{-/-} rats were exposed to maternal separation from postnatal day 2-15 either for 3 or 6 hours. Also differences in unpredictable or predictable maternal separation was tested. Finally, we also tested whether it made a difference if the mother was stressed during the maternal separation period or not. The pups were tested during adulthood for anxiety-like and cognitive behaviour in an open field (OF), elevated plus maze (EPM), home cage emergence (HCE) and novel object recognition (NOR). Results showed a genotype effect. In the OF, EPM and HCE behaviour tests an increase in anxiety-like behaviour is observed in SERT^{-/-} rats compared to SERT^{+/+} and SERT^{+/-} rats. However, no ELS effects on anxiety-like behaviour are found in this study. Furthermore, we found some GxE interaction effects in the EPM and NOR test. In the EPM SERT^{-/-}/CTRL rats, which are not exposed to ELS, show an increase in anxiety-like behaviour. This effect is less apparent in the SERT^{-/-} group when ELS is induced. Further, the SERT^{+/+}MS360 group showed an increase in anxiety-like behaviour compared to the other SERT^{+/+} groups, indicating that duration of separation also influences anxiety-like behaviour. In the NOR test the SERT^{+/+}MSU180 and SERT^{-/-}MSU180 groups show lower levels of recognition of a novel object. These results indicate an increased vulnerability of SERT^{-/-} rats to develop anxiety-like behaviour. However, a conclusive interaction between ELS, induced by maternal separation, and SERT genotype on anxiety-like behaviour is not found.

Introduction

Depressive and anxiety disorders are common at all ages. Major depressive disorder (MDD) and anxiety disorder have the highest prevalence amongst all mental and behavioural disorders (Ferrari et al., 2013). Over 4 percent of the global population suffers from a major depression and up to 4 percent suffers from an anxiety disorder (Vos et al., 2012). Several studies show that comorbidity between MDD and anxiety disorders is common (Jacobi et al., 2004; Penninx et al., 2008; Kessler et al. 1996), in the majority of this comorbidity cases MDD arose after the anxiety disorder (Graaf de et al., 2001). A diagnose of depression requires the daily prevalence of at least 5 out of 9 symptoms of depression for at least two weeks according to the *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V)* (American Psychiatric Association, 2013). These symptoms are; depressed or irritable mood, decreased interest or pleasure in activities, significant weight change, change in activity, fatigue or loss in energy, feelings of guilt or worthlessness, diminished concentration and suicidality. Point of discussion in these DSM criteria is the role of anxiety. Since anxiety often co-occurs with MDD, some see it as a symptom of MDD. However according to DSM it is a separate disorder (Zimmerman et al., 2003). Anxiety is characterized as a disorder in which levels of uncontrollably worry fluctuate, the disorder is also associated with insomnia, muscle tension and poor concentration (Zimmerman et al., 2003).

Mental disorders can be caused by a Gene x Environment (G x E) interaction (Näslund et al., 2015). G x E interactions are a combination of gene and environmental factors which influence health. G x E interactions occur when an environmental pathogen affects a person's health depending on his/her genotype or when an environmental change moderates genes and so affects health (Moffitt et al., 2005). For example, when an environmental factor that can affect mental health, but it is depended of a certain genotype. G x E interactions can have negative as well as positive effects on health. However little is known about which genes are involved, and how environmental factors increase the risk for depression (Wegener et al., 2011). An example of an environmental factor associated with an increased risk for depression could be aversive early life events such as childhood maltreatment (Heim et al., 2004). In the research of G x E interactions and mental health, genes involving in the serotonin system are of great interest. Since serotonin is one of the most important neurotransmitters influencing mental health (Stanley & Mann, 1983). Especially, genes involved with the serotonin reuptake system. Because this system is targeted by medicine used to treat depression. (Tamminga et al., 2002). These medicines called SSRI's are the most common medicine to treat depression. SSRI's function by blocking the reuptake of serotonin and increasing the serotonin levels in the brain (Sanguhl et al., 2009). The synaptic serotonin (5-HT) levels are regulated by the 5-HT transporter (5-HTT, SERT or SLC6A4) in synapses. Individuals with low 5-HT concentration whole blood levels have also low concentration 5-HT at the brain synapses which is related with enhanced negative mood and depression (Rogeness et al., 1985, Kikuchi et al., 2010). There are many variants of the SERT gene, but the human repeat length polymorphism in the promotor region of the serotonin transporter gene (5-HTTLPR) is the best studied. In previous studies three kinds of repeat sequences in the promotor region of this 5-HTTLPR are described. The short variant [S], the long variant [L] and some studies also mention the extra-large [XL]. The S-variant is associated with less mRNA expression, less serotonin binding and lower serotonin availability (Culverhouse et al., 2018; Goldman et al., 2010). The S-variant is also associated with an increased risk at developing MDD and anxiety compared to the homozygous L-variant (Lesch et al., 1996). However effects of 5-HTTLPR genotype on blood 5-HT levels are inconsistent. Some studies report an increase in 5-HT levels when the patient has at least one L-allele (Hanna et al., 1998), others did not find differences in 5-HT levels among different genotypes (Greenberg et al., 1999; Anderson et al., 2002; Betancur et al., 2002). Furthermore, there is an A/G single nucleotide polymorphism (SNP) in the long variant. The more common L_A allele is associated with the reported higher transcriptional activity, but the L_G allele functions similar to the S allele (Hu et al., 2005).

Caspi et al., (2003) were the first to take interest in this gene and found a G x E interaction between 5-HTTLPR and childhood maltreatment. Also they were able to predict depression in short allele carriers when exposed to early life stress (ELS). In the years following this finding many articles covering the relation between 5-HTTLPR genotype and depressive and or anxiety symptoms following stressful early life events have been published (Cicchetti et al., 2014; Kaufman et al. (2004,2006); Banny et al., 2013; Fergusson et al., 2011). Since the results of these studies were contradictive meta-analysis were performed. A meta-analysis of Risch et al, 2009 including 14 studies which all focused on the three 5-HTTLPR genotypes (SS, SL and LL) with a number of stressful events (0, 1,2, ≥ 3) no association between genotype and ELS was found. Also no relation between genotype and depression was found. Although the proportion of negative replication studies was significantly greater than the positive replication studies (Uher et al., 2010) another meta-analysis performed by Munafò et al. (2009) yielded no evidence that depression is a consequence of G x E interaction between 5-HTTLPR genotype and ELS. Both meta-analysis studies did not included replication studies who attempted to replicate the childhood maltreatment results of Caspi (Karg et al., 2011). Karg et al. also performed a meta-analysis and they found that 5-HTTLPR moderates a relationship between childhood maltreatment, different stressors and depression. In 2014, Sharpley and colleagues repeated the meta-analysis of Karg, but added all the studies performed the years in between. Their study provides the same relation between 5-HTTLPR and childhood maltreatment. In the most recent meta-analysis is performed by Culverhouse et al. (2018) they were not able to find a G x E interaction increasing the risk for depression.

Studies mentioned before are all human subject studies. The interaction between the serotonin transporter and ELS has also been studied in rodents. Research done in rodents result in new difficulties since they do not carry an orthologue of the 5-HTTLPR gene. Therefore a model is developed in which the serotonin transporter is knocked out by ENU -driven targeted selected mutagenesis (Smits et al., 2006). Since heterozygous knockout rats (SERT^{+/-}) show a reduced SERT expression and function they can be used as a model simulating the loss of function due to the human [S/S] or [S/L] allele 5-HTTLPR genotype. In SERT homozygous knockout rats (SERT^{-/-}), the serotonin transporter is absent, therefore SERT^{-/-} rats cannot be used as a model simulating the decrease in SERT in humans with [S/S] or [S/L] allele 5-HTTLPR genotype. Furthermore the absence of SERT results in reduced SERT expression and function, which is seen by a 40-50% decrease in SERT protein levels (Bengel et al., 1998; Homberg et al., 2007). Because of the absence of the serotonin transporter the 5-HT uptake is limited, which results in a decrease of intracellular 5-HT levels and an increase in extracellular 5-HT levels (Homberg, 2007).

SERT knockout rodents also show altered behavioural responses to stressors. For example knockout mice show more anxiety like behaviour and less exploration in elevated plus maze and open field tests (Jansen et al., 2010; Sakakibara et al. 2014). However these behavioural changes are not so apparent in SERT^{+/-} rodents compared to wild type (Holmes et al., 2003).

Adverse early life stress in animal models mirroring childhood maltreatment in humans is usually done by maternal separation, because this model is the best characterised (Haller et al. 2014). Disruptions in the mother-pup interaction in the first weeks can cause considerable changes in the neurodevelopment and behaviour of the pup (Lajud and Torner, 2015). There are several methods of maternal separation: A one-time separation of the litter from the dam for 24 hours within first week (Levine, 2000), separating the pups from the littermates and the dam for several hours per day (Ruedi-Bettschen et al., 2003). Or repeated maternal separation at stated times a day up to 6 hours during the first weeks (Plotsky et al., 1993). Another method of separation is the method of unpredictable separation; this method is based on the method of Plotsky et al. except in this case separation takes place at a different time point each day, and so making it unpredictable. This method of maternal separation can be more effective. Since the intervals of separation are applied irregular the mothers cannot anticipate the absence of their pups (Franklin et al., 2010). Methods mentioned before are all postnatal models of early life stress. Stress can also be induced prenatal by

exposing the mother to stress in the last week of the pregnancy which corresponds with the late second trimester of human pregnancy (Homberg et al., 2010). This can affect the development of the offspring by transferring stress mediators from the pregnant mother to the foetus (Huizink et al., 2004).

Interactions between early life stress and SERT genotype have also been studied in rodent models. The results of these studies differ depending on the kinds of stressors that are used as pre- and/or postnatal stressors (Houwing et al., 2017). Studies in which the stressor influences the level of maternal care show an increase in anxiety- and depressive like phenotype in SERT^{+/-} mice and rats (van den Hove et al., 2011; van der Doelen et al., 2013; Carola et al., 2008). However, previous studies only compared low levels of maternal care with high levels of maternal care. A study including normal maternal care is not yet performed. Furthermore, studies that used other early life stressors did not show an SERT genotype x early life stress interaction (Bodden et al., 2015; Kloke et al. 2013). Therefore low maternal care is used as method for ELS in this study, since this method shows the most profound and convincing behavioural effects. Also normal maternal care included as control, to observe if the interaction effects observed in previous studies are still present.

The aim of this research is to investigate whether there is a G x E interaction between SERT genotype and ELS. We expect to find an altered behavioural response in SERT heterozygotes and knockouts to ELS. To induce early life stress rats will be separated from their mothers for different time periods and under different circumstances. We suspect that rats subjected to the lower maternal care groups with SERT^{+/-} or SERT^{-/-} genotype will show an anxiety- and/or depressive-like phenotype.

Materials and Methods

Animals

Heterozygous serotonin transporter knockout male and female Wistar rats were crossbred in our own facility. Which resulted into three genotypes in the offspring: homozygous knockout (SERT^{-/-}), heterozygous knockout (SERT^{+/-}) and wildtype (SERT^{+/+}) animals. In this experiment only female offspring is used, since studies using females are scarce. After weaning animals were housed in groups of three to four. Rats in one cage belonged to the same maternal separation (MS) group but were not siblings. Cages were enriched with nesting material and wooden sticks. Animals were held in our temperature (22±1°) and humidity controlled facility in a reversed 12-hour light dark cycle (lights on at 9.00 pm). Food and water were available *ad libitum*. All experimental procedures were approved by the Groningen University Committee of Animal experiments.

Maternal separation

Offspring was subjected to early life stress by performing various forms of maternal separation (MS) (Weis et al., 2011; Franklin et al., 2010). In short, maternal separation started on either predictable or unpredictable time points and lasted for 3 hours. In addition, the effects of a longer 6 hour predictable MS period were examined. Also, it was tested if the addition of stressing the mother during the unpredictable MS period could enhance effects. Controls were separated for 15 minutes per day at predictable times. This to simulate the extra maternal care (MC) a dam gives the litter

after separation (Own et al., 2013). So, we ended up with 5 groups: controls (CTRL), maternal separation at predictable time points for 3 hours (MS180), for 6 hours (MS360) and unpredictable 3 hour maternal separation without maternal stress (MSU180) or with maternal stress (MSUS180). The 3 or 6 hour separation took daily place during the dark cycle and started at postnatal day 2 (PND2) and lasted until PND15. Litters were placed in clean cages on heating mats during the separation, visual and olfactory contact was not possible. During separation, the dams stayed in their home cages and had access to food and water, the pups from one litter remained together. During PND2-8 temperature was set at $32\pm 1^{\circ}\text{C}$, while temperature was set at $28\pm 1^{\circ}\text{C}$ during PND9-15. The dams of the MSUS180 group were unpredictably and randomly exposed to two types of maternal stress at unexpected times: a 5 minute forced swim test (FST) or a 20 minute restraint stress (RS) in a Plexiglas tube. An example of a maternal separation and stress scheme is provided in supplementary table S1. At PND7, PND14 and PND21 the pups were weighed and the cages cleaned, the mothers were weighed from PND1 until PND16. At PND21 the pups were weaned and reared in cages of 3-4 rats per cage. Each caged contained rats of the same sex and treatment, but the rats were from different mothers. Table 1 gives a clear overview of the number of rats per genotype and treatment.

Table 1

	CTRL	MS180	MS360	MSU180	MSUS180
SERT +/+	10	9	9	7	8
SERT +/-	9	9	9	9	8
SERT -/-	10	9	9	7	8

Behavioral testing

Behavioral testing was started when the offspring (F_1) reached adulthood, 12 weeks old. In this study only female offspring was used. During all tests, the experimenter was blind to treatment. The behaviors displayed during the tests were monitored with video tracking (Ethovision and Observer, Noldus Information Technology Wageningen the Netherlands). Each test animal underwent the tests mentioned in table 2, with at least a weekend between two tests. All tests took place under dim light. A decrease in anxiety behavior is found when rats are in estrous (Goes et al, 2015; Ramos-Ortolaza et al., 2017), therefore before starting or after finishing the test animals were tested for estrous. Rats in estrous did not participate in the test that day, or were excluded from the results. The open field (OF), elevated plus maze (EPM), home cage emergence (HCE) and novel object recognition (NOR) tests were used as measures for anxiety-like behavior.

Table 2

Time schedule different behavioral tests.

Age	
PND2-PND15	MS/MC scoring
PND21	Weaning
12 weeks	Open field
13 weeks	Elevated plus maze
16 weeks	Home cage emergence
17 weeks	Novel object recognition

19 weeks	Sacrifice
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Open field (OF)

Before starting the open field test estrous was measured, rats in estrous were excluded from testing that day and tested another day. Females were placed in a square 100 cm x 100 cm x 50 cm box. The box was placed in a room with dim light, in the center the light strength was 12.6 lux and at the walls it was between 9.2 and 10.9 lux. Animals were placed in the right, bottom corner of the box and were allowed to explore for 10 minutes. Time spent in the center (60 cm x 60 cm) of the OF and the distance moved was recorded with an automated tracking system (Ethovision, Noldus Information Technology). Between two rats the OF is cleaned with 70% ethanol to remove all traces of the previous rat.

Elevated plus maze (EPM)

Female rats were tested in the elevated plus maze under dim light, which consists of two opposite open arms(2,5 lux) and two opposite closed arms(0,25 lux). The subjects were placed in the center (1,25 lux) of the EPM facing the left open arm of the elevated plus maze (each arm is 10 cm x 50 cm x 40 cm). The subjects were allowed to explore the EPM for 5 minutes. The EPM was raised 50 cm above the ground. Time spent in open and closed arms and the total distance moved were quantified using Ethovision. Before testing estrous was measured and after testing the EPM was cleaned with 70% ethanol.

Home cage emergence (HCE)

Prior to the HCE, animals were individually housed for 12 days. During the HCE the home cage of the test subject was moved to the test room and placed in an open field arena. Then the home cage was opened and a metal grid was bend and placed in the cage. Latency to escape the home cage was measured using a stopwatch. 10 minutes were provided to escape the home cage. After testing estrous was measured.

Novel object recognition (NOR)

The novel object recognition test was performed in a rectangular open arena (100 cm x 50 cm x 40 cm). The test took place on two consecutive days and consisted of two phases: habituation and testing. At day 1 a 5 minute habituation in the arena without objects took place. After exactly 24 hours the subject was placed back in the arena and another 3 minute habituation took place. This followed by a 3 minute testing phase with two similar objects were placed in the arena. Then after 45 seconds without objects two dissimilar objects were placed in the arena one familiar and one unfamiliar object at the same location. The location (left or right) of the unfamiliar object was alternated between animals to control for possible spatial bias. The subject was free to explore the arena again for 3 minutes (figure 1). In the test two types of objects were used: (1) a transparent glass bottle filled with flour, available in duplicate. (2) An opaque purple plant sprayer, which was used as unfamiliar object. Using the scoring system Observer (Noldus Information Technology) the behavior of the subject during the two 3 minute testing phases was scored. Freezing, grooming and exploration duration and frequency of the objects were the behaviors that were scored. Animals that did not explore both objects for at least 3 seconds during the habituation or testing phase were

excluded from analysis. After the second testing phase estrous was measured. Between two rats the arena and objects were cleaned with 70% ethanol.

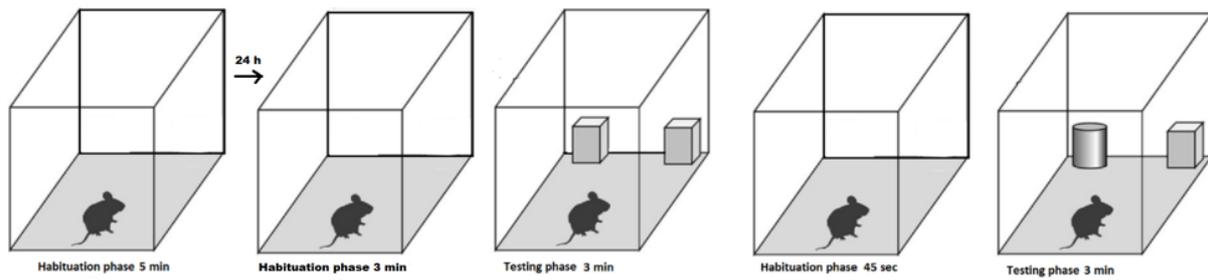


Figure 1: Novel object recognition test experimental setup. The cuboids represent the transparent glass bottles filled with flour (familiar object) and the cylinder represents the opaque purple plant sprayer (unfamiliar object). During the testing phase the unfamiliar object can be placed on the left as well as the right side.

Statistical analysis

First, data was checked for parametric distribution. If non-parametric, data was transformed to fit a normal distribution. All behavioral parameters were analyzed by using a two way (multifactorial) ANOVA (genotype x treatment) followed by a Fishers LSD post hoc to correct for multiple comparisons. Significance was set at $p < 0.05$ for all tests. Error bars represent SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Results

Early life stress has been reported to play a role in the development of anxiety and depressive like symptoms (Laugharne et al., 2010). In addition, disturbances in the SERT system are known to contribute in the development of anxiety and depression symptoms (Houwing et al., 2017). In this study we investigated if there is G x E interaction between SERT genotype and ELS. And if so if we can find a model in which a combination of SERT genotype and induced ELS results in a greater change of developing anxiety like behavior. We investigated if the female offspring of the 15 different groups showed anxiety-like behavior, using classical anxiety paradigms.

OF

In the OF test we observed that there were no significant genotype, treatment or genotype x treatment interactions for the time spent in the center of the OF arena (data not shown). We also analyzed the first five minutes of the time spent in the OF, here no significant results were found either (Figure 2A). However, a genotype effect was found in the total distance moved in the first five minutes of the test [$F(2, 114) = 3.648, p = 0.029$]. Knockouts covered a significantly greater distance than heterozygotes and wild types SERT^{-/-} (n = 42) vs. SERT^{+/-} (n = 44), $p = 0.003$; SERT^{-/-} vs. SERT^{+/+} (n = 43), $p = 0.015$) (Figure 2B).

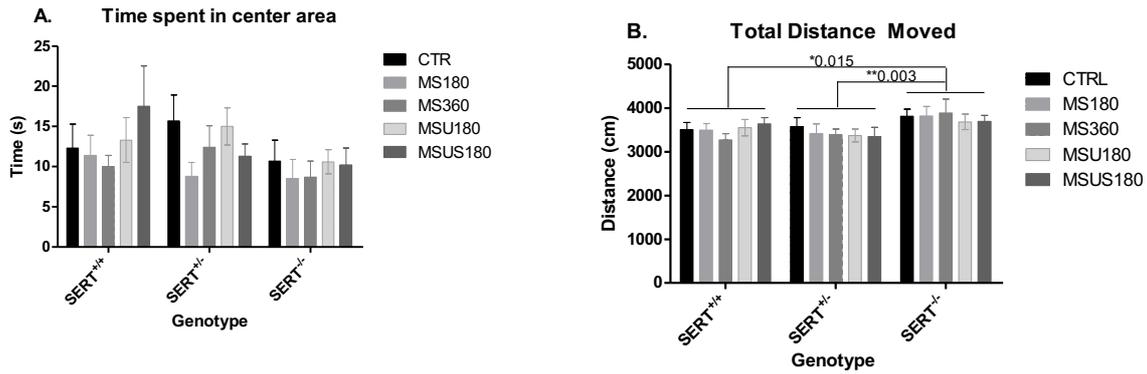


Figure 2: Open field test. Female offspring. **(A)** Time spent in the center area of the OF. [SERT^{+/+}: CTRL, n = 10; MS180, n = 9; MS360, n = 9; MSU180, n = 7; MSUS180, n = 8. SERT^{+/-}: CTRL, n = 9; MS180, n = 9; MS360, n = 9; MSU180, n = 9; MSUS180, n = 8. SERT^{-/-}: CTRL, n = 7; MS180, n = 9; MS360, n = 8; MSU180, n = 9; MSUS180, n = 9.] **(B)** Distance moved in the first 5 minutes per genotype in centimeter (cm). * $p < 0.05$; ** $p < 0.01$. Bar graphs represent mean values \pm SEM.

EPM

Another commonly used behavioral test to measure anxiety is the elevated plus maze (EPM). In this test we measured the time the rats spent in the open arm of the EPM. The more time they spend in the open arm the less anxious they are. We observed a genotype effect [$F(2, 113) = 12.9, p = 0.000$], with SERT^{-/-} rats spending significantly less time in the open arm compared to SERT^{+/+} and SERT^{+/-} animals. [SERT^{-/-} (n = 42) vs SERT^{+/-} (n = 45) $p < 0.000$ and for SERT^{-/-} vs SERT^{+/+} (n = 41) $p < 0.000$]. (Figure 3A). Furthermore we observed a treatment x genotype interaction [$F(8, 113) = 2.11, p = 0.041$]. *Post hoc analysis* showed that this effect was significant for MS360-SERT^{+/+}, this group spent significantly less time in the open arm compared to CTRL-SERT^{+/+}, MS180-SERT^{+/+} and MSU180-SERT^{+/+}. Also MS180-SERT^{+/-} spent significantly less time in the open arm compared to MSUS180-SERT^{+/-}, furthermore the CTRL-SERT^{-/-} group spent significantly less time in the open arm compared to MS360-SERT^{-/-}, MSU180-SERT^{-/-} and MSUS180-SERT^{-/-} but also to CTRL-SERT^{+/-} and CTRL-SERT^{+/+}. Other significant differences were that MS360-SERT^{-/-} spent less time in the open arm compared to MS360-SERT^{+/+} and MSUS180-SERT^{-/-} to MSUS180-SERT^{+/+}. Again we observed that SERT^{-/-} rats were more active than SERT^{+/+} and SERT^{+/-} [$F(2, 113) = 5.879, p = 0.004$ SERT^{-/-} vs SERT^{+/-} $p = 0.002$ and for SERT^{-/-} vs SERT^{+/+} $p = 0.006$] (figure 3B).

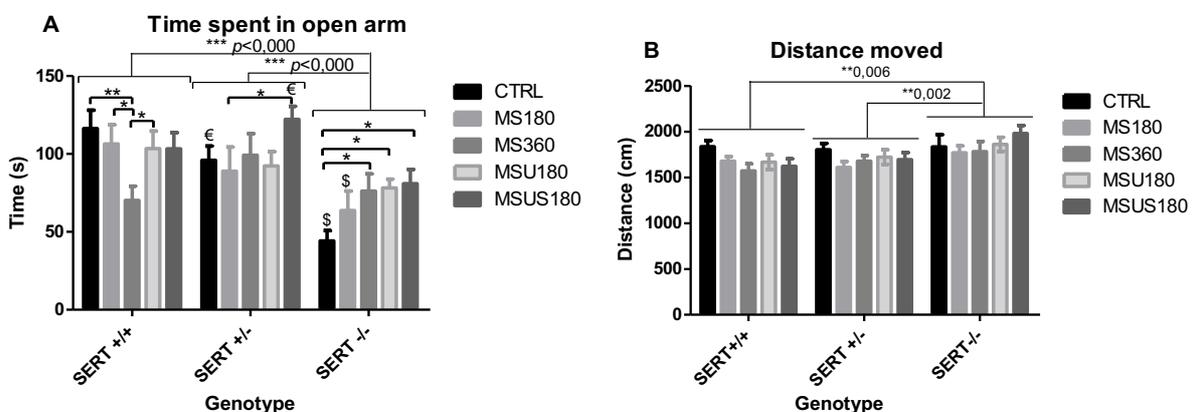


Figure 3: Elevated plus maze. Female offspring. **(A)** Time spent in the open arm of the EPM. [Wild type SERT^{+/+}: CTRL, n = 10; MS180, n = 9; MS360, n = 9; MSU180, n = 7; MSUS180, n = 6. Heterozygous SERT^{+/-}: CTRL, n = 9; MS180, n = 9; MS360, n = 9; MSU180, n = 9; MSUS180, n = 9. Knockout SERT^{-/-}: CTRL, n = 7; MS180, n = 10; MS360, n = 8; MSU180, n = 7; MSUS180, n = 9.]

= 10. $\$p < 0.05$ between SERT^{-/-} vs SERT^{+/-}, $\epsilon p < 0.05$ between SERT^{+/-} vs SERT^{-/-}. (B) Distance moved in the EPM per genotype in centimeter (cm). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Bar graphs represent mean values \pm SEM.

HCE

Next we tested the latency to escape the home cage. This task is used to measure risk and exploratory behaviors. Before entering a new environment rodents typically take time to evaluate the potential danger of the new environment (Weis et al. 2011). The longer a rodent takes to explore the new environment the more anxious it is. In the HCE test we found a genotype effect [$F(2, 87) = 4.28, p = 0.017$] SERT^{-/-} rats showed significant higher escape latencies than SERT^{+/-} rats. Additionally, a trend was observed in the escape latency of SERT^{+/-} animals. However no genotype x treatment interaction was found. Also no significant treatment effects of latency to escape the home cage were observed (Figure 4).

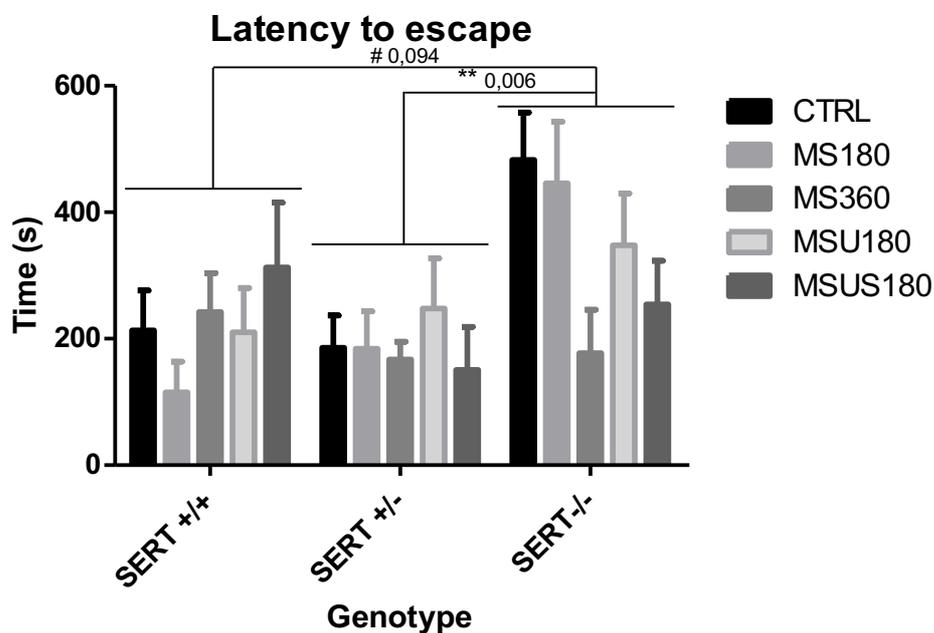


Figure 4: Home cage emergence test. Female offspring. Latency time to escape the home cage. [SERT^{+/-}: CTRL, n = 8; MS180, n = 4; MS360, n = 8; MSU180, n = 6; MSUS180, n = 6. SERT^{+/-}: CTRL, n = 6; MS180, n = 7; MS360, n = 8; MSU180, n = 7; MSUS180, n = 8. SERT^{-/-}: CTRL, n = 6; MS180, n = 6; MS360, n = 7; MSU180, n = 8; MSUS180, n = 7. #0.05 < $p < 0,1$; ** $p < 0.01$. Bar graphs represent mean values \pm SEM.

NOR

We next tested the impact of treatment and genotype on object recognition. In the NOR test, we observed that all groups spent more time exploring the unfamiliar object than the familiar object [$t(112) = 21.487, p < 0.001$]. An interaction effect between treatment and genotype was observed [$F(8, 98) = 2.36, p = 0.023$]. Treatment effects [$F(4, 98) = 1.67, p = 0.163$] and genotype effects [$F(2, 98) = 0.37, p = 0.691$] on the recognition index were not found. Further analyzing the observed treatment x genotype interaction we found that MSU180-SERT^{-/-} group spent significantly less time exploring the unfamiliar object compared to the other SERT^{-/-} groups (figure 5). MSU180-SERT^{-/-} also spent less time exploring the unfamiliar object compared to MSU180-SERT^{+/-}. MSU180-SERT^{+/-} had a significantly lower recognition index than MSU180-SERT^{+/-} and MS180-SERT^{+/-}. Furthermore some

trends were observed, the MSU180-SERT^{+/+} group showed a lower recognition index compared to CTRL-SERT^{+/+} and MS360-SERT^{+/+}. Other observed trends were between MS180-SERT^{+/+} and MSUS180-SERT^{+/+}, MS360-SERT^{+/+} and MS360-SERT^{+/-} and finally MS360-SERT^{+/-} and MS360-SERT^{-/-}. Total exploration time of the objects, freezing and grooming behavior data are also analyzed, but no interactions effects are found (data not shown).

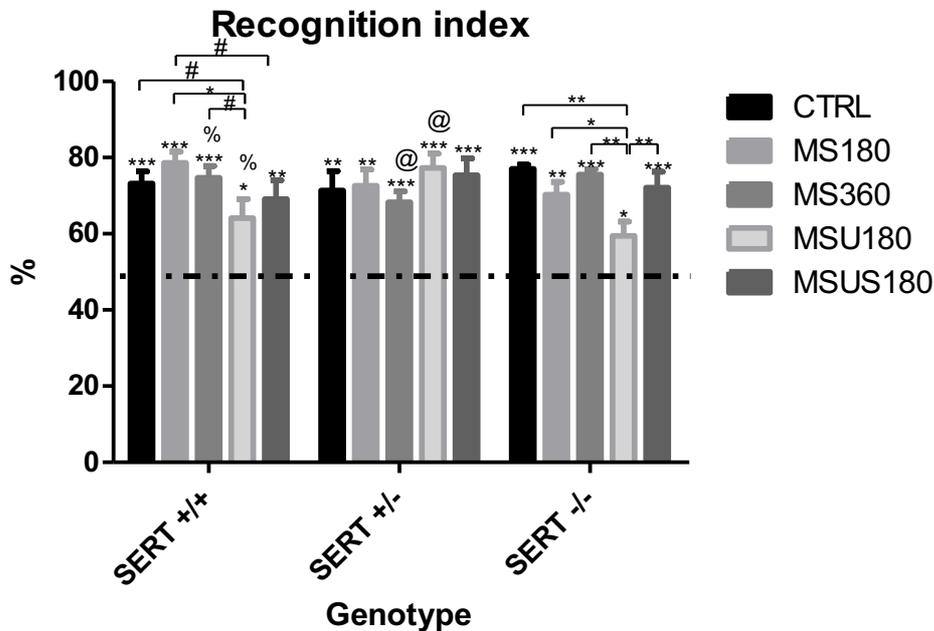


Figure 5: Novel object recognition (NOR) test. Female offspring. Recognition index, recognition of the new object as percentage of the familiar object. [SERT^{+/+}: CTRL, n = 10; MS180, n = 9; MS360, n = 7; MSU180, n = 6; MSUS180, n = 6. SERT^{+/-}: CTRL, n = 8; MS180, n = 8; MS360, n = 9; MSU180, n = 8; MSUS180, n = 8. SERT^{-/-}: CTRL, n = 5; MS180, n = 6; MS360, n = 7; MSU180, n = 8; MSUS180, n = 8. All the groups spent more time exploring the new object over the old. %*p* < 0.1 between SERT^{+/+} vs SERT^{+/-}, @*p* < 0.1 between SERT^{+/-} vs SERT^{-/-}. #0.05 < *p* < 0,1; **p* < 0.05; ***p* < 0.01; ****p* < 0.001. Bar graphs represent mean values ± SEM.

Discussion

Early life stress is known to alter behavior in rodents, primates and humans. In our study we searched for a rat model in which SERT genotype and a MS treatment interaction could induce an anxiety-like phenotype. In this study three variants of the SERT gene in rats are included; SERT^{+/+}, SERT^{+/-} and SERT^{-/-}. SERT^{-/-} rats show altered brain serotonin levels which are associated with changes in behavior (Kalueff et al., 2010). MS is often used as a form of early life stress. In this study we had five variants of MS treatment; a CTRL, MS180, MS360, MSU180 and MSUS180 group. All of these treatment groups previously showed to have influence on rodent behavior (Weis et al., 2011; Franklin et al., 2010; Millstein & Holmes, 2007). We measured anxiety-like behavior to determine which combination of SERT gene variation and treatment results in an alteration of behavior.

Our results support evidence that the knockout of the SERT gene leads to an increase of anxiety-like behavior in female rats compared to rats with SERT^{+/+} or SERT^{+/-} genotype. In other words a genotype effect is found. Furthermore, in the EPM and NOR tests genotype x treatment interactions on anxiety were found. However, these effects of induced early postnatal stress in combination with SERT genotype on behavior are not conclusive. For example, the groups which showed anxiety-like

behavior in the EPM test were different from the ones in the NOR test. Furthermore, the SERT^{-/-} genotypes showed an increase in activity compared to SERT^{+/-} and SERT^{+/+}. We did not find any effects of pre- or postnatal induced early life stress.

This study confirms that at least in the EPM and HCE the SERT^{-/-} rats show an increase in anxiety-like behavior. In the EPM the SERT^{-/-} rats spent more time in the closed arm of the maze, also the SERT^{-/-} rats took longer to escape the home cage compared to SERT^{+/+} and SERT^{+/-}. Therefore the knockouts seem more anxious than the wild types and heterozygous'. The same behavioral alteration is found in SERT^{-/-} mice (Holmes et al., 2003). These mice also spent less time in the open arm of the EPM, they also showed less exploration and performed worse in emergence tests. Jansen et al., 2010 also studied anxiety-like behavior of mice with SERT gene variation; they did not find differences in time spent on the open arm of the EPM between different SERT genotypes. However in a dark-light emergence test they did find a latency to emerge in SERT^{-/-} rats. In the OF test we did not observe a genotype effect, this in contrast with the results of Sakakibara. In this study SERT^{-/-} mice showed increased anxiety-like behavior in the OF as well as in the EPM (Sakakibara et al. 2014). Taken together, our findings suggest a genotype effect on anxiety in rats. Interestingly, we found that SERT^{+/-} rats did not exhibit behavioral alterations. The performance of SERT^{+/-} rats in the behavioral tests did not significantly differ from that of SERT^{+/+} rats. Although heterozygote knockouts are lacking one allele of the SERT gene, which is known to cause a decrease in 5HT-reuptake compared to SERT^{+/+} (Montanez et al. 2003). Indicating, that the gene function can be restored by the presence of at least one allele of the SERT gene.

Behavioral differences caused by changes in maternal care which are found in previous studies are not found in ours. Mice induced to low maternal care showed increased anxiety related behavior (Carola et al. 2008). They showed a decreased OF and EPM exploration (Caldji et al., 2000; Carola et al., 2008). Also studies in which maternal care is altered by separating the pups from the mother an increase in anxiety-like behavior is found (Franklin et al., 2010 and Weis et al., 2011). Weis separated the pups from the mother at unexpected time points, also some of the mothers were stressed during this time period of separation. The MSUS pups showed a mild increase in risk taking behavior in the OF and EPM, which is indicated as abnormal behavior. The latency to enter unfamiliar areas of MSUS pups however tended to be lower than the controls (Weis et al., 2011). However in our study in none of the behavioral tests a treatment effect is found. In none of the maternally separated groups an increase in anxiety-like behaviour was observed. This is surprising since it is known that the mother serves as a primary link between the developing rat-pup and its environment (Levine, 1975). However the altered behaviour by MS in previously mentioned studies was mostly shown by males. Franklin et al. 2010 tested only males in the F1 generation, which is the generation we look at in our study. Also in the OF and EPM the clearest behavioural alterations were in males, however females did spent more time in the open arm of the EPM (Weis et al, 2011). There are also some other studies which did not observe effects of postnatal early life stress on behaviour. In a study performed by Kloke et al. 2013 maternal care was altered by altering the early environment. They used three different early environments: soiled beddings of unfamiliar males (adverse), normal nesting conditions (standard) and communal nesting (enriched). The offspring was tested for anxiety behaviour, but no differences in anxiety were found.

In the EPM and NOR test we observed genotype x treatment interactions. Some groups were significantly more anxious than the others. For example the SERT^{-/-}CTRL group did not spent much

time in the open arm of the EPM, indicating that the animals in this group are more anxious. The SERT^{-/-} group which is not exposed to ELS, shows a great increase in anxiety like behaviour. Which is also shown in EPM tests performed by Holmes et al. (2003) and Sakakibara et al. (2014). However this effect of SERT^{-/-} genotype on behaviour is less apparent when ELS is induced. It seems that induced ELS in SERT knockouts makes the rats less anxious. A similar effect of ELS on behaviour is found in the study of Weis et al. (2011). Here MSUS pups spent more time in the open arm of the EPM. In the EPM the SERT^{+/+}MS360 group spent less time in the open arm, indicating that a long period of separation causes an increase in anxiety-like behaviour. The SERT^{+/+}CTRL and SERT^{+/-}MSUS180 groups on the other side spent much more time in the open arm, indicating that these groups are less anxious. In the NOR test also genotype x treatment interactions are found. However if you compare the results from the EPM test with the NOR test, no similarities in groups are found. For example, the SERT^{-/-}CTRL group in the EPM seems to be the most anxious group, however in the NOR test there is no data indicating that group being anxious. In the NOR test the SERT^{+/+}MSU180 and SERT^{-/-}MSU180 groups show lower levels of recognition of a novel object, indicating that they have a lower memory performance. The NOR test is usually used as a measure for cognitive capabilities. Nevertheless Behan et al., 2011 did show that prenatally stressed females showed increased depressive-like behaviour with cognitive deficits. Therefore you could say that alterations in behaviour showed by the SERT^{+/+}MSU180 and SERT^{-/-}MSU180 groups are a consequence of the observed genotype x treatment interaction. Time exploring the objects during the test can also be a measure of anxiety. However we did not observe any significant differences in exploring time between groups.

Looking at all behavioural tests we cannot point out one particular experimental group which shows an increased anxiety-like behaviour caused by a GxE interaction. Although these findings do confirm that anxiety behaviour in rats can be affected by treatment and genotype. This is in accordance with studies performed by Bodden et al., 2015 and Kloke et al. 2013, who also observed genotype and ELS effects on anxiety-like behaviour.

In both the OF and EPM we observed an increase in activity of the SERT^{-/-} rats. The knockouts covered a greater distance during the testing phase. This increase of activity can indicate a decrease of anxiety since the rats are showing an increase in exploration. This increase in activity contradicts the results of Olivier et al. (2008) and Kalueff et al. (2007). The first study showed no differences in activity, the second even showed a hypoactivity of the SERT^{-/-} genotype compared to the SERT^{+/+}. This hypoactivity was observed both EPM and OF tests. So, the observed increase in exploration in our study could mean that SERT^{-/-} rats are less anxious than SERT^{+/-} and SERT^{+/+}. However Franklin et al. 2010 suggested that observed increase in exploration could also reflect a decrease in behavioural control rather than an overall decrease in anxiety. Also behavioural control is regulated by the serotonergic system, which is neurochemically changed due to the SERT^{-/-} genotype (Homberg et al. 2007).

There are some limitations that can be addressed in further research. Firstly, the rats used in this study underwent 7 different behavioural tests in a short time period, which may have influenced the behavioural response. The short time period between the tests could have such an impact on all the rats that also the controls experienced anxiety and therefore no significant differences were found between the groups. In the future a 2-week interval between tests could be taken into consideration. Secondly, in this study the animals were divided in two pilots. As a result, the animals

of both pilots were born in different times of the year. However since the animals were housed in climate controlled rooms this should not be of influence. For further research it could be interesting to include also males in the research. Because females seem to be less sensitive to early life stress compared to males (Kikusui and Mori, 2009). A limiting factor in some tests of this experiment was the group size. Some groups only existed out of 5 subjects after oestrous measurements, this because the animals in oestrous affected the results in such a way that alteration in anxiety behaviour were less clear. This specifically in the HCE test, in which animals were tested for oestrous after performing the test. Another interesting point for further research is if the females used in the experiment transmit behaviour alterations to their offspring. It is already shown that unpredictable maternal separation can alter behaviour across generations in adult mice (Franklin et al., 2010) However experiments across generations including animals with SERT gene variation and that are maternally separated from the pups are not yet performed.

In conclusion our results do not fully support the theory of Caspi et al., 2003 that individuals with a 5-HTTLPR s-allele (in our study the SERT+/- genotypes) and exposed to ELS have a higher change of developing an anxiety or depression disorder. However studies performed in humans not always show this interaction (Culverhouse et al., 2018; Munafò et al., 2009). Also studies in rodents do not always confirm this interaction (Bodden et al., 2015; Kloke et al. 2013). However it is clear that there is an interaction between SERT gene variation and ELS, but the mechanisms underlying this interaction appear to be so complex that further investigation is necessary (Houwing et al., 2017).

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Supplementary table 1: MS and maternal stress schedule during first postnatal week

PND	MS180 time	MS360 time	MSU180 time	MSUS type	MSUS Time stress
1	9.30-12.30	9.30-15.30	13.30-16.30	RS	14.00-14.20
2	9.30-12.30	9.30-15.30	12.00-15.00	FST	12.15-12.20
3	9.30-12.30	9.30-15.30	9.30-12.30	RS	10.00-10.20
4	9.30-12.30	9.30-15.30	11.00-14.00	FST	13.30-13.35
5	9.30-12.30	9.30-15.30	13.30-16.30	RS	16.00-16.20
6	9.30-12.30	9.30-15.30	12.00-15.00	RS	11.30-11.50
7	9.30-12.30	9.30-15.30	9.30-12.30	FST	14.40-14.45