Serotonin transporter x early life stress interaction on sociability and ethanol intake

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Abstract

Problem drinking that becomes severe is given the medical diagnosis of alcohol use disorder (AUD) and is a serious public health problem worldwide. It is unknown who will become diagnosed with AUD, but genes do play an important role. One of the genes linked to AUD is the serotonin transporter gene (5-HTT). The 5-HTT protein is a key regulator of 5-HT tone (extracellular). Variation in the serotonin (5-HT) system may contribute to the risk of alcohol dependence. This polymorphism is characterized by a long (L) and short (S) allele. The short allele is linked to decreased pre-synaptic 5-HTT number and functionality. A recent meta-analysis in human populations showed a correlation between homozygous S allele carriers and AUD. There are several environmental factors which also contribute to the development of addictive behavior, including early life stress and social factors. Rodent studies showed that separations of intact litters increased ethanol consumption and/or preference in adult male rats compared to shorter periods of MS. In addition, rodents lacking the 5-HTT were more vulnerable for anxiety- and depression-related behaviors. However, little is known about the interaction of early life stress and serotonin transporter genotype.
In this study the interactive effects of serotonin transporter deficiency, which mimics the less efficient human variant of the serotonin transport (S allele of the 5-HTTLPR polymorphism), and early life stress were studied on ethanol intake and social behavior, in rats.
Serotonin transporter knockout rats (SERT $^{-/-}$), serotonin transporter heterozygous (SERT $^{+/-}$) and serotonin transporter wildtype rats (SERT $^{+/+}$) were separated from their dams for 360 minutes once a day from postnatal day 2 till 16. To test their social behavior a three-chamber test was performed and social interaction was measured. Thereafter animals were presented with 20% ethanol in their home cages, on an intermittent-every-other day schedule. Volumes consumed were determined to assess the ethanol intake.
On a social level, SERT $^{-/-}$ rats had significant lower preference for interaction with a novel rat compared to SERT $^{+/+}$ rats and SERT $^{+/-}$ rats. In addition, early life stress increased ethanol intake in male SERT $^{+/-}$ and SERT $^{-/-}$ rats significantly. In Females, a trend toward an effect of genotype on ethanol intake was found. With SERT $^{-/-}$ rats drink more compared to SERT $^{+/+}$ rats. Our data also revealed that females drank more ethanol compared to males, which may be due to hormones.
In conclusion, our results show that male maternally separated SERT $^{-/-}$ rats drink more ethanol. This effect is not seen in females. This might suggest that SERT $^{-/-}$ genotype increases the vulnerability to stress stimuli in males. Suggesting that females are more resilient for early life stress than males. Future research must be done into the underlying mechanisms of the interaction between early life stress and SERT genotype, and the sex differences that are found.

Key words: AUD, serotonin, 5-HTT Polymorphism, sociability, sex-differences

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1. Introduction

Heavy alcohol (ethanol) consumption remains a serious public health problem worldwide. Problem drinking that becomes severe is given the medical diagnosis of alcohol use disorder (AUD). Approximately 14 percent or 32 million adults in the United States ages 18 and older had AUD in 2012 (Grant et al, 2015). To be diagnosed with AUD, individuals must meet certain criteria outlined in DSM-V (The Diagnostic and Statistical Manual of Mental Disorders). There are 11 criteria mentioned in the DSM-V, anyone meeting any two of the 11 during the same 12-month period receives a diagnosis of AUD. Although the severity of AUD (mild, moderate or severe) is based on the number of criteria met (Tuithof et al, 2013). For example AUD patients will drink more or longer than intended, experience withdrawal symptoms when not drinking (such as trouble sleeping, shakiness, anxiety, depression, restlessness) or give up on activities that were important or interesting to them in order to drink (Vonghia et al, 2014).

In 10 to 20% of consumers, chronic alcohol use contributes to medical complications including damage to organs and immune functions. Despite the high prevalence and costs to society, treatment options for these disorders are limited in number and efficacy (Spanagel, 2009). In addition, factors that increase the risk of developing alcohol addiction include psychiatric disorders, such as anxiety and major depressive disorders (Spanagel, 2009). Furthermore, antisocial personality disorder is associated with a high degree of alcoholism (Schubert et al, 1988).

While a great majority of the people worldwide uses alcohol, only a few develop alcohol use disorder. Twin and adoption studies implicate the importance of genetic influences on the development of alcohol use disorder (Mcgue, 1999). Genetic differences do likely play a role. One of the genetic differences which is studied in humans is a polymorphism in the serotonin transporter gene (5-HTT). Variation in the serotonin (5-HT) system may contribute to the risk of alcohol dependence. The 5-HTT protein is a key regulator of 5-HT tone as it re-uptakes 5-HT from the synaptic cleft back into the presynaptic neuron where it can be degraded or retained for future release. The 5-HT transporter thereby controls the extracellular concentration of 5-HT (Murphy et al, 1998).

A functional polymorphism is identified in the promotor region of the 5-HTT gene (5-HTTLPR) which is characterized by a long (L) and a short (S) allele. The short allele is linked to decreased pre-synaptic 5-HTT number and functionality, resulting in less efficient serotonin reuptake and increases in synaptic 5-HT levels (extracellular), as compared to the long allele (Lesch, 2007; Nordquist et al, 2010; Canli and Lesch, 2007). Recent meta-analysis done in human populations showed a correlation between the homozygous S allele polymorphism and AUD (Oo et al, 2016). Previous research has also shown that the concentrations of 5-hydroxyindoleactic acid (5-HIAA), the first serotonin degradation product, were lower in the cerebrospinal fluid of alcoholics than in non-alcoholics. This is in line with animal models of alcohol abuse. For example, rats bred for alcohol preference had lower levels of serotonin and its metabolites in various brain regions compared to nonpreference rats (Lovinger, 1997). However, results of research done on the interaction of 5-HTTLPR and alcohol use or misuse are so far contradictory.

A study done in hundred ten human males with alcohol dependence concluded that the short allele of the 5-HTTLPR was unrelated to alcohol dependence (Gorwood et al, 2000). On the other hand, a single photon emission computed tomography of alcohol dependent patients showed reduced levels of the serotonin transporter in the brain. The reduction in availability of brainstem serotonin transporters was significantly correlated with lifetime alcohol consumption (Heinz et al, 1998).

Serotonin plays a role in the positive reinforcement of alcohol abuse (Lovinger, 1997). Serotonin alters the dopaminergic system because it stimulates the activity of dopaminergic neurons in the ventral tegmental area (VTA), this causes an increased dopamine release. The dopaminergic neurons in the VTA

are connected with brain areas which mediate rewarding effects. So serotonin activates these neurons and therefore it can reinforce alcohol-drinking behavior (Lovinger, 1997).

However, alcohol use disorder is not only the result of an individual's genetic make-up. In fact, there are several environmental factors which contribute to the development of addictive behavior. Prenatal alcohol exposure, prenatal stress and severe stressful life events all play a role in the vulnerability to AUD. A prenatal stress experiment often used in rodents is maternal separation (MS). Results from currently used MS protocols are not uniform, this is also due to differences in the protocols. However, studies consistently show that longer separations of intact litters increase ethanol consumption and/or preference in adult male rats compared to shorter periods of MS. Research of Palm et al (2013) showed that a separation of 360 minutes increases total ethanol intake and preference over time in adult male rats compared to control (MS of 15 minutes). A study of Barr et al (2003) studied Rhesus Macaques that were peer-reared (PR) and as a control mother-reared (MR) macaques. The peer-reared monkeys were separated from their mothers at birth and hand reared in a neonatal nursery for the first 30 days of life (early life stress). This study investigated the relationship between early-life stress, serotonin transporter genotype and sensitivity to ethanol. They found that monkeys heterozygous for the serotonin transporter polymorphism (l/s) had an increased ethanol sensitivity compared to monkeys with the l/l genotype (wildtype). Being peer-reared increased the sensitivity to ethanol but only among the l/s monkeys. These results were consistent with findings in human studies where human subjects with the l/l genotype were associated with a low level of response to alcohol and alcohol consumption (Barr et al, 2003).
So early life stress is a major environmental risk factor for alcohol use disorder. A biological explanation for this can be that prenatal and postnatal stress can alter the activity of the hypothalamic-pituitary axis (HPA) (Schepis et al, 2011). Long-lasting changes in glucocorticoid levels may occur that influence mesolimbic dopaminergic activity and reinforcement processes.

5-HT is a key modulatory neurotransmitter and has been implicated in the pathophysiology and treatment of anxiety and mood disorders (Olivier et al, 2008). Previous research of Olivier and colleagues showed that rats lacking the SERT are more vulnerable for anxiety- and depression-related behaviors. For instance, SERT $^{-/-}$ rats spend significantly less time in the center of an open-field test. In addition, SERT $^{-/-}$ rats spend significantly less time on the open arms in an elevated plus maze. Both test results indicate more anxiety-like behavior in SERT $^{-/-}$ rats compared to SERT $^{+/+}$ rats. Further, SERT $^{-/-}$ rats drink significantly less sucrose compared to SERT $^{+/+}$ rats. This is being indicative for anhedionia, a core symptom of depression. Besides effects on anxiety- and depression-related behaviors a study of Homberg et al (2008a) showed that SERT $^{-/-}$ rats display lower social play behavior compared to SERT $^{+/+}$ rats. These results are in line with meta-analysis done in humans (Sharpley et al, 2014; Manufo et al, 2009).
Not only serotonin transporter genotype has an effect on behavior. Research of Gianchino and colleagues shows that maternal separation reduces emotionality, assed by a social interaction test, in mice (Gianchino et al, 2007).

In the present study we investigated the interactive effects of serotonin transporter deficiency, which mimics the less efficient human variant of the serotonin transport (S allele of the 5-HTTLPR polymorphism), and early life stress on ethanol intake and social behavior, in rats.
We first investigated the effects of early life stress and serotonin transporter variability (SERT $^{+/+}$, SERT $^{+/-}$, SERT $^{-/-}$) on sociability?
Next we investigated the effects of early life stress and serotonin transporter variability on ethanol intake.

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We hypothesized the serotonin transporter knockout rats (SERT $^{-/-}$) to be the least social, compared to serotonin transporter heterozygous (SERT $^{+/-}$) and serotonin transporter wildtype rats (SERT $^{+/+}$). In addition, we expect that MS will further reduce social behavior in SERT $^{-/-}$ and SERT $^{+/-}$ rats, while SERT $^{+/+}$ rats may be resilient to the effect of MS.

With regard to ethanol intake, we hypothesized that MS will increase ethanol intake and that ethanol intake will be strongest in SERT $^{-/-}$ and SERT $^{+/-}$ rats. In addition, we expect SERT $^{-/-}$ and SERT $^{+/-}$ rats to drink more ethanol compared to SERT $^{+/+}$ rats, with a genotype-dose dependent pattern.

2. Material and Methods

2.1 Animals

Subjects were 69 Wistar rats, serotonin transporter $^{+/+}$ (SERT $^{+/+}$), serotonin transporter $^{+/-}$ (SERT $^{+/-}$) and serotonin transporter $^{-/-}$ (SERT $^{-/-}$). This experiment was done in male and female rats. Table 1 shows all experimental groups and the number of animals per group. This study was carried out in batches with the aim of eventually having 12 animals per group. This report discusses the results of batch 1 and 2 (together).

Rats were bred in the department of behavioral neuroscience at the University of Groningen. Rats were weaned on postnatal day 21 (PND21). They were socially housed in cages with 3 or 4 rats per cage in our vivarium (± 21°C, 40-60% humidity). They were kept on a 12 hour light/dark cycle (lights off: 10:00 A.M). After the sociability test rats were individually housed with a 12 hour light/dark cycle (lights off: 7:00 A.M). Cage bedding was refreshed weekly and water and standard pelleted RHMB chow were freely available in the home cages. Body weights were recorded 3 times a week throughout the study. All experiments were performed in accordance with the Dutch Laws on Animal Welfare and European regulations and were approved and checked by the Animal Experimentation Committee of the University of Groningen.

Genotype	Treatment	Sex	# animals
SERT $^{+/+}$	MS	Male	10
		Female	9
	Control	Male	1
		Female	7
SERT $_{+/-}$	MS	Male	11
	Control	Male	10
SERT $^{-/-}$	MS	Male	7
		Female	4
	Control	Male	4
		Female	6
Total			69

Table 1. Experimental groups and number of animals. During the 3-chamber test there were 10 groups, as listed above. During the ethanol experiment all groups were divided into water (control) and ethanol groups. This led to 20 groups.

2.2 Maternal separation

In the maternally separated group, the pups were separated from their dams for 360 minutes once a day from postnatal day 2 (separations cycles were performed between 9:00 and 15:00 pm) and continued for 14 days. Pups were removed as complete litters from the nest, placed into a cage with heating pads in an adjacent room to prevent mothers from hearing ultra-sound vocalizations. The temperature of their cages was maintained at 32 ± 1˚C during the first week (PND2-8) and 28 ± 1˚C during the second week (PND9-15). In the control group, pups were removed as a litter to cages and handled for 15 minutes. After 15 minutes the pups were reunited with their dam. On PND 7, 14 and 21 all pups were weighted and sexed on PND7. Weaning took place on PND 21 and animals were housed by sex and treatment group.

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2.3 Three chamber test

A three-chamber social apparatus (3 times 40 cm (120 cm) × 80 cm) was used as the testing arena (Reilly et al, 2015 and Moy et al, 2004). Testing was conducted under pink light (2.5 lux) during the dark period of their light–dark cycle, approximately 1 hour following lights out. One hour prior to testing, estrus stage of the females was measured. The females performed the social test on days that they were not in the estrous stage.

The test started by placing the experimental animal in the middle chamber of the apparatus, with the doors to the two other side chambers closed. This habituation (Reilly, Weeks et al. 2015) was done for 5 minutes. After this habituation a same-sex stimulus animal (SERT [+/+] males and SERT [+/+] and SERT [+/-] females) was placed in one of the cylindrical grids (diameter: 22cm) located in the outer corner of the side chambers. The doors to both side chambers were removed so the animal could explore all three chambers. This was done for 10 minutes. The bars in the grids allowed rats for nose-to-nose investigation but did not permit further contact (Reilly et al, 2015). Time spent at the center, social side, non-social side were scored with Observer XT 10.5 (Noldus [®], Wageningen, the Netherlands). In addition, time spent at the social- and non-social grid was also scored. Using these data the preference for social interaction has been calculated.

2.4 Free choice ethanol

The animals were housed individually and put on a ethanol (20%) paradigm in their home cages, on an intermittent-every-other day schedule. Maternally separated or control rats were initially offered a bottle with ethanol for 7 h/day for three days/week (Monday, Wednesday and Friday) for one month. Food and water were freely available. Subsequently, the rats had access to ethanol for 24 h/day for three days/week (Monday, Wednesday and Friday) for another month. Volumes consumed were determined by weighing the bottles prior to and after each session, and from these volumes the ethanol intake (g/kg) was calculated. The positions of the bottles was alternated to prevent side bias. 24 hours after the start of the last ethanol session blood samples were collected from the tail vain. 48 hours after the start of the last ethanol session rats were sedated with CO_2 and sacrificed by decapitation. Brains were collected and stored at -80 for future gene expression analyses.

2.5 Data analysis and statistics

GraphPad Prism V5.0 (GraphPad Software, San Diego, CA) and IBM SPSS statistics were used for statistical analysis. An ANOVA for repeated measures was done to test significance on ethanol intake. When significant, an independent sample t test was used to determine in which weeks the ethanol intake was significant. For the sociability a multivariate linear ANOVA test was done to test significance. To detect differences between genotypes, treatment and sex, a one-way ANOVA was used. When appropriate a LSD post-hoc test was used to detect the differences between genotypes. Results are presented as mean ±SEM values. A P value of less than 0.05 was considered significant.

3. Results

3.1 Sociability

To investigate whether serotonin genotype influences sociability rats were tested in a 3-chamber test when they were 10 weeks old.
The following variables were measured: time spent at the center, social side, non-social side, social-grid and non-social grid. Using these data the preference for social interaction was calculated: preference is the time spent at social grid divided by time spent at the social-grid plus time spent at non-social grid x 100%.

A significant genotype effect was found for social interaction ($F_{(1,2)}=4,25$, $p<0,05$), time spent in the center ($F_{(1,2)}=4,12$, $p<0,05$) and social preference ($F_{(1,2)}=4,23$, $p<0,05$). In addition, there was a trend for time spent in the social chamber ($F_{(1,2)}=3$, $p=0,058$). There was no effect of treatment and sex on all measured variables. Therefore, results were split in genotypes and not in treatment groups and sex. An one-way ANOVA showed only a strong tendency for social preference ($F_{(1,2)}=3,031$, $p=0,055$). In the other parameters, further testing revealed no significant differences between the individual groups. Figure 1 shows the effects of SERT genotype on social preference. LSD post-hoc test revealed that SERT [-/-] rats have a significantly lower social preference compared to SERT [+/+] rats (p=0,038) and SERT [+/-] rats (p=0,033).
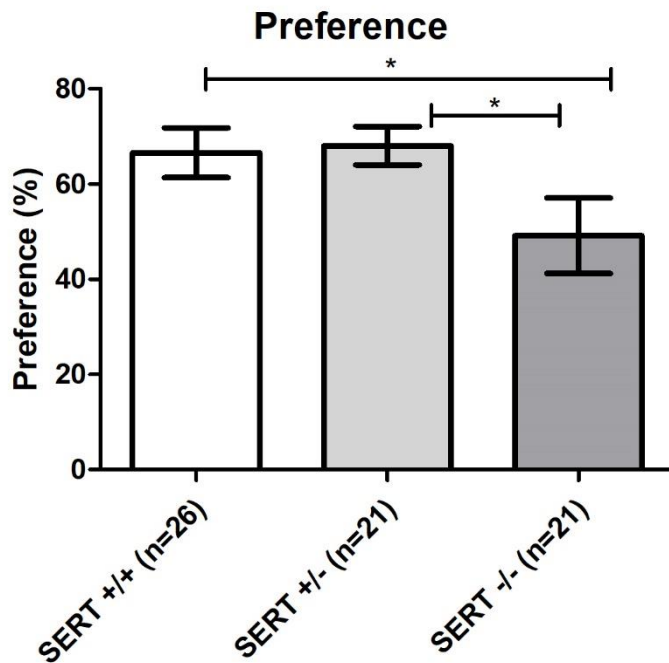

figure 1. 3-chamber test results from male and female, MS and control rats, separated in SERT [+/+], SERT [+/-] and SERT [-/-] rats. Behavior was observed for 10 minutes. Results are shown as mean ± SEM. * p<0,05. The figure shows mean ± SEM for social preference. SERT [+/-] rats have a significantly lower social preference compared to SERT [+/+] rats (p=0,038) and SERT [+/-] rats (p=0,033).

3.2 Ethanol intake

As mentioned in the material and method section, the free choice ethanol experiment consisted of two periods. The first four weeks, rats were exposed to one bottle of ethanol (20%) for 7 h/day for three days/week (Monday, Wednesday and Friday). The last four weeks, the rats had access to ethanol for 24 h/day for three days/week (Monday, Wednesday and Friday).

A significant difference in ethanol intake between male and female rats was found ($F_{(1,7)}$=3,64, $p<0,005$). Therefore the results of the ethanol intake are split per sex.

Males

In males, a significant effect of maternal treatment on ethanol intake ($F_{(1,7)}$=3,69, $p<0.001$). In figure 2, the results for male rats are separated on genotype. This was done to investigate whether there was an effect of treatment on ethanol intake in all genotypes.

Figure 2a shows no significant differences between control handled SERT [+/+] rats and maternal separated SERT [+/+] rats. However, there is only 1 animal in the control group here, so no conclusions can be drawn from this result. Figure 2b shows a trend in week 5 and 7 for SERT [+/-] rats that were maternally separated to drink more ethanol in weeks 5 ($t_{(16,45)}$=1.89, $p=0.077$) and 7 ($t_{(16)}$=1,96, $p=0,067$), compared to control SERT [+/-] rats. Figure 2c shows that that SERT [-/-] rats that were maternally separated drink significantly more ethanol in week 8 ($t_{(9)}$=-2,64, $p<0,05$), compared to control SERT [-/-] rats.

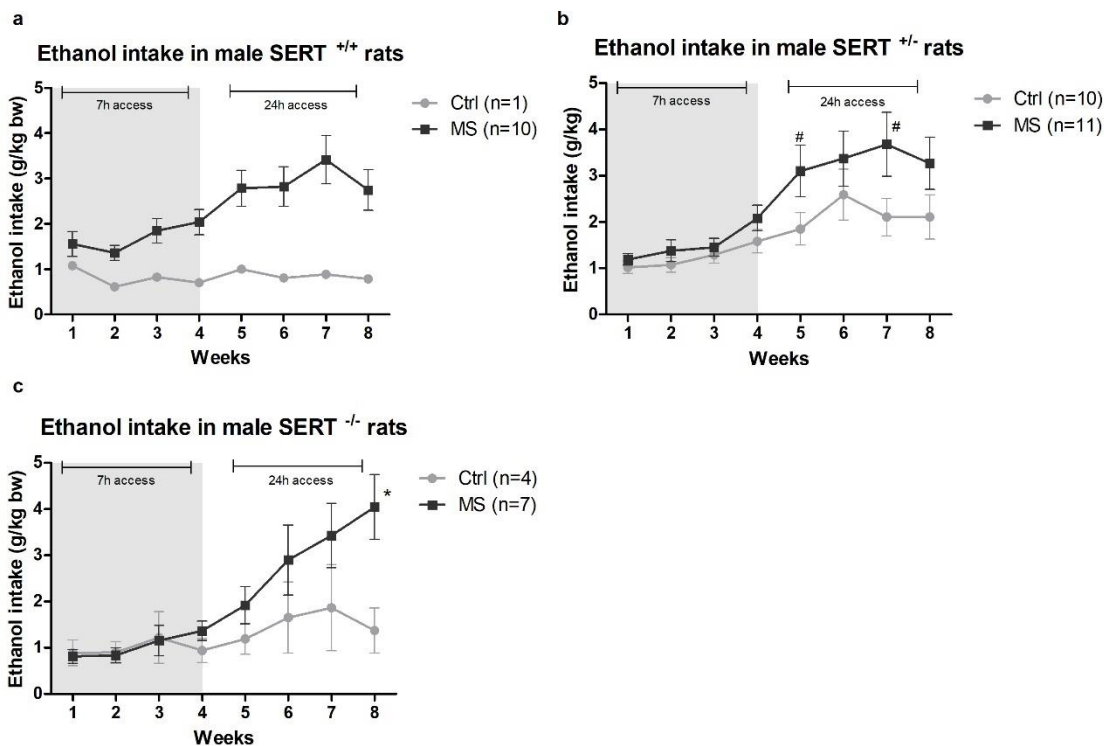### Treatment effect on ethanol intake in males

Figure 2. Ethanol consumption results from male SERT [+/+], SERT [+/-] and SERT [-/-] rats. Results are shown as mean ± SEM for ethanol intake in gram per kilograms of bodyweight over 8 weeks. * $p<0,05$, # $P<0,08$. **a.** Mean ± SEM for ethanol intake of maternal separated and control handled SERT [+/+] male rats. No significant differences were revealed. **b.** Mean ± SEM for ethanol intake of maternal separated and control handled SERT [+/-] male rats. There is a trend in week 5 and 7. SERT [+/-] rats that are maternally separated drink more ethanol in weeks 5 ($t_{(16,45)}$=1.89, $p=0.077$) and 7 ($t_{(16)}$=1,96, $p=0,067$), compared to control SERT [+/-] rats. **C.** Mean ± SEM for ethanol intake of maternal separated and control handled SERT [-/-] male rats. SERT [-/-] rats that are maternally separated drink significantly more ethanol in week 8 ($t_{(9)}$=-2,64, $p<0,05$), compared to control SERT [-/-] rats.

Figure 3 shows that there was no genotype effect on ethanol intake in control handled and maternal separated male rats.

Genotype effect on ethanol intake in males

a

Ethanol intake in male control rats

Legend:
- Wildtype (n=1)
- Heterozygote (n=10)
- Knockout (n=4)

b

Ethanol intake in male MS rats

Legend:
- Wildtype (n=10)
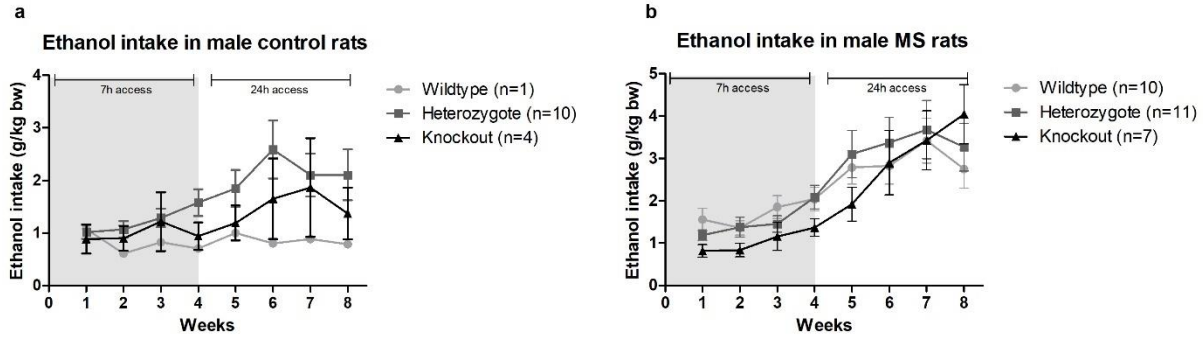- Heterozygote (n=11)
- Knockout (n=7)

Figure 3. Ethanol consumption results from male control handled and maternal separated rats. Results are shown as mean $\pm$ SEM for ethanol intake in gram per kilograms of bodyweight over 8 weeks. **a.** Mean $\pm$ SEM for ethanol intake of SERT $^{+/+}$, SERT $^{+/-}$ and SERT $^{-/-}$ male rats. No significant differences were revealed. **b.** Mean $\pm$ SEM for ethanol intake of SERT $^{+/+}$, SERT $^{+/-}$ and SERT $^{-/-}$ male rats. No significant differences were revealed.

Females

In females, a trend was found toward an effect of genotype on ethanol intake ($F_{(1,7)}$=1,82, p=0.088). No significant differences were revealed in both control handled female rats (figure 4a) and maternal separated female rats (figure 4b).

Genotype effect on ethanol intake in females

a

Ethanol intake in female control rats

Legend:
- Wildtype (n=7)
- Knockout (n=6)

b

Ethanol intake in female MS rats
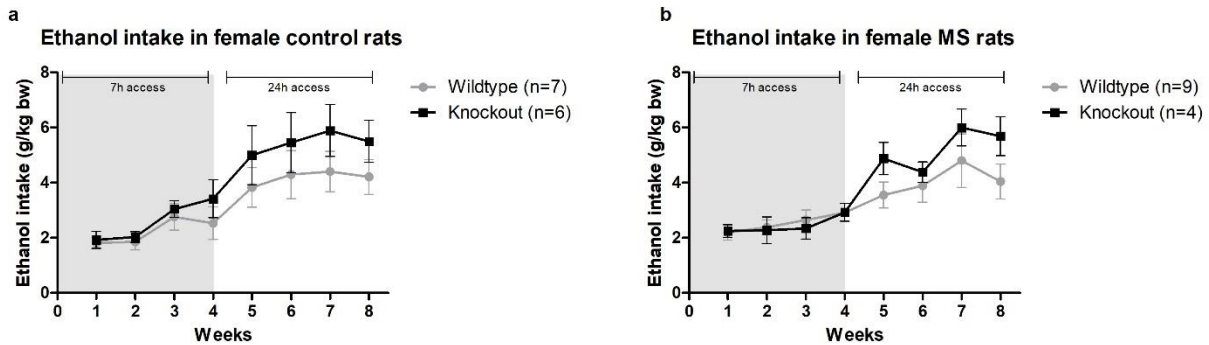

Legend:
- Wildtype (n=9)
- Knockout (n=4)

Figure 4. Ethanol consumption results from female control handled and maternal separated rats. Results are shown as mean $\pm$ SEM for ethanol intake in gram per kilograms of bodyweight over 8 weeks. **a.** Mean $\pm$ SEM for ethanol intake of SERT $^{+/+}$ and SERT $^{-/-}$ female rats. No significant differences were revealed. **b.** Mean $\pm$ SEM for ethanol intake of SERT $^{+/+}$ and SERT $^{-/-}$ female rats. No significant differences were revealed.

Figure 5 shows that there is no treatment effect on ethanol intake in SERT [+/+] and SERT [-/-] female rats.

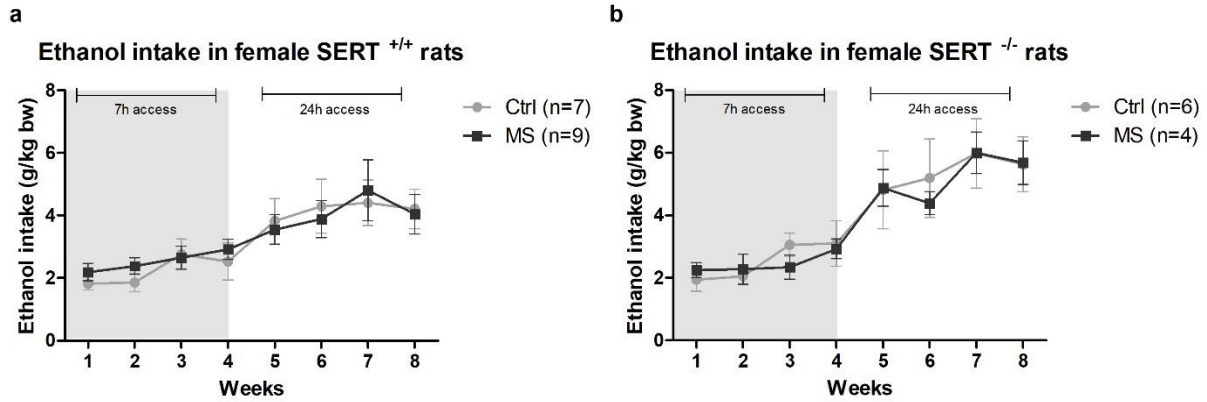## Treatment effect on ethanol intake in females

Figure 5. Ethanol consumption results from female SERT [+/+] and SERT [-/-] rats. Results are shown as mean ± SEM for ethanol intake in gram per kilograms of bodyweight over 8 weeks. **a.** Mean ± SEM for ethanol intake of control handled and maternal separated female SERT [+/+] rats. No significant differences were revealed. **b.** Mean ± SEM for ethanol intake of control handled and maternal separated female SERT [-/-] rats. No significant differences were revealed.

4. Conclusion and discussion

Sociability

The first aim of this research was to investigate the interaction effects of early life stress and serotonin transporter genotype on sociability. No effect of early life stress on sociability was found, which was against our expectations. We expected MS to reduce social behavior, especially in SERT [-/-] and SERT [+/-] rats. This is because MS is probably not stressful enough to cause changes in wildtype (SERT [+/+]) rats. In the literature there are conflicting results about the effect of maternal separation on social behavior. It is important to note that there is a lot of difference in the age of rodents in the literature. A study done in adult (PND 92) male and female mice showed that the female mice show more social behavior after maternal separation during a social interaction test. The maternally separated male mice showed no difference in social behavior with regard to control males (Bondar et al, 2018).

A study done in rats showed different results (Holland et al, 2014). Maternal separation had an effect on the social behavior of juvenile female rats. They had less nose-to-nose contact in a social interaction test, compared with non-separated (PND 25) female rats. It also toke significantly longer before they made contact with the other rat during the social interaction test. There was no difference in juvenile male rats. When this test was repeated during adulthood (PND 40), the results were different. Then there was no longer any difference between control female rats and maternally separated female rats. However, then there was a difference in male rats. Male maternally separated rats needed more time before they made the first contact during a social interaction test, compared to control male rats. However, the total duration of the nose-to-nose contact of the MS male rat was equal to that of the control male rats. These results are in line with our findings, as we did not find any difference between MS and control animals in total duration the social interaction.

The results of the juvenile (PND 25) female rats are in agreement with a study by Farrell and colleagues. They also looked at social behavior of juvenile male and female rats. They saw that maternal separation increased the latency to make nose-to-nose contact in females but not males (Farrell et al, 2016). Although in our study, juvenile behavior was not assessed, we did not find any differences in social interaction during adulthood as a result of maternal separation.

However, there is an effect of serotonin transporter genotype on sociability. As our results showed that SERT [-/-] rats had a significantly lower preference for the social grid compared to the SERT [+/-] and SERT [+/+] rats. The SERT [-/-] rats therefore had less social interaction compared to the SERT [+/+] and SERT [+/-] rats. This is in line with our expectations. Research done in mice also showed that SERT [-/-] mice show less social behavior during a social interaction test (Kaleuff et al, 2007). In addition, when SERT [-/-] mice had to perform a resident-intruder test, they also showed less aggressive behavior, compared to SERT [+/+] and SERT [+/-] mice. It took longer before they attacked the intruder for the first time, they attacked less and the intensity of the attack was lower (Holmes et al, 2002).

Like SERT [-/-] mice, SERT [-/-] rats also showed reduced aggressive behavior in the resident-intruder test (Homberg et al, 2007). Other groups studying social behavior in SERT [-/-] mice showed that SERT [-/-] mice also show a strong reduction in their social approach behavior (Moy et al, 2009). These mice had a reduction in the time they were in the department where a stimulus mouse was present (Page et al, 2009). This corresponds with the result of our SERT [-/-] rats who spend less time in the social-chamber. In addition, these rodent findings are in line with research done in rhesus macaques. Rhesus macaques with one copy of the 5-HTTLPR short allele spent less time staring at face than non-face images and spent less time looking in the eye region of faces (Watson et al, 2009).

Altogether, the SERT [-/-] phenotype seems less social compared to SERT [+/+] and SERT [+/-] animals. However, anxiety is an important factor to keep in mind when interpreting the results of social testing.

As described in the introduction, SERT $^{-/-}$ mice and rats were more anxious than SERT $^{+/+}$ and $^{+/-}$ mice and rats. This might play a role in translating the results of social behavior tests (Kaleuff et al, 2010). A study done in social groups of mice showed that SERT $^{-/-}$ mice are not less social than SERT $^{+/+}$ and SERT $^{+/-}$ mice. They looked at the social behavior of mice group-housed in their home cage. Lewejohann and colleagues argued that studies performed on SERT $^{+/+}$, SERT $^{+/-}$ and SERT $^{-/-}$ rodents often involve tests performed with rats unknown to the rest rat and in an novel environment, where anxiety can play a major role (Lewejohann et al, 2010). In our experiment we had 11 rats who did not move at all, spending all their time in the center. From those 11 rats, the biggest group was the male maternally separated SERT $^{-/-}$ group, with 4 rats. This might indicate anxiety in the maternally separated SERT $^{-/-}$ group, however this parameter is not enough to conclude this. In future research it has added value to also measure other parameters during the three-chamber test. Such as total mobility and freezing behavior. It would also be an option to retest the rats the next day with the same stimulus rat to rule out an effect of social anxiety (Kaidanovich-Beilin et al, 2011).

Ethanol intake

Our second aim of this research was to investigate the interaction effects of early life stress and serotonin transporter genotype on ethanol intake.

Overall, in all groups of rats there was an overall effect of ethanol intake over the 8 weeks. This means, that all groups of rats drink more ethanol as the weeks progress. This only does not apply to the SERT $^{+/+}$ control group in males. It is important to say that this groups contains only 1 rat and we therefore cannot draw conclusions from those data. This increase in ethanol intake can partly be explained by the fact that during the first four weeks the rats only had access to ethanol for 7 hours a day. While during the last four weeks, the rats had access to ethanol for 24 hours a day, suggesting that a longer access to ethanol increase the intake of ethanol.

When focussing on ethanol intake, it was found that females drank more ethanol compared to males. In previous research of Torres and colleagues it was also shown that the rewarding effects of ethanol were enhanced in female rats (Torres et al, 2014). An important finding in that study was that ethanol had no rewarding effects in ovariectomized rats. As they drank significantly less ethanol than male and control female rats. This suggests that the presence of ovarian hormones is important for the reward of ethanol in females. The role of estrogen in the ethanol rewarding process in women is also supported by the fact that ovariectomized rats drank a lot more ethanol when given estrogen (Ford et al, 2004). Preclinical research in rodents also showed that females had a larger voluntary intake of ethanol in a 2-bottle choice procedure compared to males and females without ovarian hormones (Torres et al, 2014). This is in line with our results.

Our results showed an effect of early life stress on ethanol intake in males. However, we did not find an effect of early life stress on ethanol intake in females. We expected that MS would increase ethanol intake and that this effect was strongest in SERT $^{-/-}$ and SERT $^{+/-}$ rats. This because we expected SERT $^{+/+}$ rats to be resilient to the effects of MS (Barr et al, 2003). Our findings in male rats are in line with the hypothesis. SERT $^{-/-}$ rats that were maternally separated drank more ethanol in week 8, compared to control handled SERT $^{-/-}$ rats. Maternally separated SERT $^{+/-}$ rats also had an increase in ethanol intake compared to control handled SERT $^{+/-}$ rats. However, this was a trend. While in males the results perfectly match our hypothesis, females showed a different pattern, where no differences in ethanol intake were found when comparing control handled and maternally separated rats (in both SERT $^{-/-}$ and SERT $^{+/+}$ groups).

These results are in line with previous research. In research of Roman and colleagues (2004), three groups of rats were used. The first group was separated for 15 minutes (MS15), the second group was

separated for 360 minutes (MS360) and the third group was not handled at all (control). Their results showed that female rats did not differ in mean ethanol intake between the three groups (Roman et al, 2004). Other research, with the same protocol showed that male MS15 rats had a lower voluntary ethanol intake compared to male MS360 rats and male control rats (in all ethanol concentrations: 2%, 4%, 6% and 8%). The maternal separation of 15 minutes thus showed a protective effect in male rats compared to MS360 (Ploj et al, 2003). Our control rats also underwent a maternal separation of 15 minutes. It could therefore be that our male rats also have this protective effect. In addition, the male MS360 rats showed an increase in the voluntary ethanol intake compared with the male control rats at 8% (Ploj et al, 2003). In the present study a 20% ethanol solution was used, indicating that maternal separation of 360 minutes combined with a high ethanol concentration increases ethanol intake, but as our data shows, only in vulnerable animals (SERT $^{-/-}$ rats).

As discussed above, previous research showed differences in the effects of maternal separation on ethanol intake between males and females. Possible explanations for this can be different separation-induced neurochemical effects, hormone-dependent effects or sex-dependent mother-infant behavior during the postnatal period (Roman et al, 2004). In our data, in male rats there was an effect of early life stress on ethanol intake while there was no effect seen in females. Studies on various models of early life stress in rodents showed sex differences. Male rodents who experienced early life stress showed impaired behavioral performance in a third of cases while this was only 25% in females (Loi et al, 2017). Recent data suggested strong sex difference in effects of early life stress, with females being more resilient to stress in general than males (Walker et al, 2017). This is in line with our data. Walker and colleagues mention that, like in our study, the bases for these sex differences are not clear. Whether external factors like sex-specific differences in maternal care or sex hormones of the pups are involved, or whether other early life factors like nutrition and metabolism or epigenetics play a role remains to be demonstrated (Walker et al, 2017).

Researchers also studied the link between early life stress and the development of alcohol addiction in humans. In these studies the focus was mainly on the effects of stressors such as parental divorse, sexual abuse, family violence, economic adversity and parental death and mental illness (Green et al, 2010). The age in which the stressor takes place is of great importance. Research showed that stress during an age younger than 5 years is related to larger psychopathology during adulthood (Kaplow and Widom, 2007). The fact that stress during the first years has the greatest effect can be explained by the sensitivity of cortisol activity. This system is particularly sensitive to the quality of nurturing during the first years of life (Gunnar and Donzella, 2002).
For example, a study was done in 1362 male and female native Americans. This study showed that for men who have been exposed to multiple forms of abuse, the individuals had a threefold greater risk of AUD. Women who had been exposed to multiple forms of abuse had a sevenfold greater risk of AUD (Koss et al, 2003). However, not all research is in line with these results. For example, another study showed that maltreatment before the 11th year of life was not the predictor for the development of alcohol addiction (Kaplow and Widom, 2007).
So early life stress can lead to permanent changes, including a greater chance of developing an addiction. However, a large proportion of the children who experienced severe early life stress did not develop psychopathy. This implies that other factors such as gene x environment interactions and family relationships are of importance (Enoch, 2011).

When looking at the effects of serotonin transporter genotype on ethanol we see no effect in males. In addition, in females a trend was found towards an effect of serotonin transporter genotype on ethanol intake. The results in female are in line with our expectations. As we expected SERT $^{-/-}$ and SERT $^{+/-}$ rats to

drink more ethanol compared to SERT [+/+] rats, with a genotype-dose dependent pattern. However, in females we did not include SERT [+/-], therefore we cannot say anything about the genotype-dose dependent pattern.

As mentioned in the introduction, there has been a lot of contradiction in the results of research into the interaction between the 5-HTTLPR and alcohol misuse. Some of the studies showed no association between serotonin transporter genotype and ethanol intake (Lamb et al, 2013, Gorwood et al, 2000). Other research showed a connection between the short allele of the 5-HTTLPR and alcohol intake (Oo et al, 2016, Lamb et al, 2014, Heinz et al, 1998). However, it is important to say that most of the studies are all done in male rodents and/or human male patients.

We expected our SERT [-/-] rats to drink more compared to the SERT [+/-] and SERT [+/+] rats. This because, as mentioned in the introduction, research in humans showed that the reduction in availability of serotonin transporters in the brain was significantly correlated with lifetime alcohol consumption (Heinz et al, 1998). However, other research showed that SERT [-/-] mice drank even less ethanol than SERT [+/+] mice in a free-choice procedure of progressive ethanol self-administration. So in this research inactivation of the 5-HT re-uptake leads to a reduction in ethanol intake in SERT [-/-] mice (Kelei, 2003). This result is in line with research of Lamb and Daws (2013). They showed that male SERT [-/-] mice have a lower breaking point when responding to 12% ethanol under a progressive ratio schedule, compared to SERT [+/-] and SERT [+/+] rats. The SERT [-/-] mice also tended to stop working for ethanol at lower fixed ratio values than the SERT [+/-] or SERT [+/+] mice (Lamb and Daws, 2013).

It is currently unclear how 5-HT affects positively reinforced behavior (Kaleuff et al, 2007). However, recent findings of a study using MRI showed markedly altered corticolimbic pathways in SERT [-/-], biased towards posterior areas. Some of these areas are directly involved in the reward circuit, pointing to specific modulation of this circuit by the altered lifelong serotonin tone (Bearer et al, 2009).

When looking at research done on other drugs of abuse, research by Homberg and colleagues showed that SERT [-/-] rats were more sensitive to cocaine (2008b). The intravenous cocaine self-administration was enhanced in SERT [-/-] rats. SERT [-/-] rats self-administered higher amounts of cocaine than SERT [+/+] and SERT [+/-] rats (with a 0.3 mg / kg cocaine dose).

Research of Daws and colleagues (2006) suggested that ethanol inhibits the clearance of 5-HT from the extracellular fluid. When the SERT actively reuptakes 5-HT, it compensates for the inhibition of 5-HT clearance from the extracellular fluid. Ethanol then only provides a modest increase in 5-HT. The effects of ethanol on extracellular 5-HT levels would be significantly enhanced by the loss of the 5-HTT function (Daws et al, 2006). So they suggested that ethanol increases extracellular levels of 5-HT. However, later research of Verheij and colleagues (2014) showed that 5-HT levels are not increased after cocaine intake, as they are already increased to a ceiling level in SERT [-/-] rats. The levels of dopamine and noradrenaline do increase due to cocaine use. This suggests that the 5-HT levels in SERT -/- rats are already so high that drugs (such as cocaine) can no longer increase them (Verheij et al, 2014). It therefore seems unlikely that ethanol will result in higher 5-HT levels in SERT -/- rats, like Daws and colleagues suggest, although this needs to be confirmed.

SERT genotype x early life stress

Research was also done into the interaction between MS and SERT genotype in general. Our research showed that SERT genotype is important for the response to MS. Only SERT [-/-] rats showed significant effects of MS. So it is possible that SERT genotype may determine stress response in rodents (reviewed in Houwing et al, 2017). Research from Li and colleagues (1999) showed that the stress-induced response in plasma ACTH levels after a saline injection were higher in SERT [+/-] mice compared to SERT [+/+] mice. This may suggest that the sensitivity to stressful stimuli is increased in SERT [+/-] mice (Li et al, 1999). However,

further changes of the HPA axis are limited. No differences were found in the most important components of the HPA axis between SERT [+/+] and SERT [+/-] rodents (Houwing et al, 2017). In addition, SERT genotype x early life stress interactions have also been studied in the hippocampus of female SERT [+/+] and SERT [+/-] mice. This study showed that SERT genotypes and early life stress exposure influences the expression of levels of genes involved in the mitogen-activated protein kinase signaling pathway and neurotrophic signaling. This research suggested that this can ultimately also contribute to increasing susceptibility to stress (van den Hove et al, 2011).

In general, it is still unclear what the underlying mechanisms are that trigger the interaction between SERT genotype and early life stress. This subject is therefore important for future research.

SERT genotype effects on ethanol intake

Our results showed that both male and female SERT [-/-] rats are less social compared to SERT [+/-] and SERT [+/+] rats. In addition, SERT [-/-] rats (female) had a higher ethanol intake compared to control rats. Research has also been done into the link between antisocial behavior and the development of alcohol addiction in humans. For example, a longitudinal study was done in 2586 pupils. This study explored the causal effects of alcohol misuse and antisocial behavior between the ages 11 and 15. Overall, their results showed that antisocial behavior was the main predictor of alcohol misuse (Young et al, 2007). Another longitudinal study done in 4354 females and 3984 males showed that antisocial behavior was predictive of alcohol use during mid-to late adolescence (from age 13 to 17) (Cho et al, 2014).

However, as mentioned before, anxiety could also have played a role during the 3-chamber test. SERT [-/-] rats are more anxious than control animals (Olivier et al, 2008). Research has shown that high levels of anxiety and anxiety-related characteristics (i.e. low novelty seeking, low resistance to punishment) can predict addiction-like ethanol drinking in mice.

In conclusion our results showed that male maternally separated SERT [-/-] rats drank more ethanol. This could be explained by the effects of stress. The HPA axis is the response system to environmental challenges and opportunities (including stressors) in many species. Stressful stimuli activate neurons or a specific region of the hypothalamus, the paraventricular nucleus (PVN). Those neurons secrete releasing hormones, such as corticotropin-releasing factor (CRF). The releasing hormones act on the anterior pituitary to promote the secretion of adrenocorticotropic hormone (ACTH) into the blood circulation. Circulating ACTH leads to an increase in glucocorticoids (reviewed in Houwing et al, 2017).

A recent review article gave an overview of the effects of early life stress on various key components of the HPA axis (Houwing et al, 2017). This review showed that different components of the stress system were changed after early life stress, in SERT [+/-] and SERT [-/-] rodents. This could provide the differences that we have found in ethanol intake. However, in order to be able to say this with certainty, further research on the levels of different HPA components in the brains of our rats should be considered.

5. References

1. Barr C.S., Newman T.K., Becker M.L., Champoux M, Lesch K.P., Suomi S.J., Goldman D, Higley J.D. 2003. Serotonin transporter gene variation is associated with alcohol sensitivity in rhesus macaques exposed to early-life stress. Alcohol Clin Exp Res. 27(5): 812-7.
2. Barr C.S, Newman T.K, Lindell S, Shannon C, Champoux M, Lesch K.P, Suomi S.J, Goldman D, Higley J.D. 2004. Interaction between serotonin transporter gene variation and rearing condition in alcohol preference and consumption in female primates. Arch Gen Psychiatry. 61(11):1146-52.
3. Bearer E.L., Zhang X, Janvelyan D, Boulat B, Jacobs R.E. 2009. Reward circuitry is perturbed in the absence of the serotonin transporter. NeuroImage. 46:1091-1104.
4. Bondar N.P., Lepeshko A.A., Reshetnikov V.V. 2018. Effects of early-life stress on social and anxiety-like behaviors in adult mice: sex-specific effects. Behavioural neurology. 2:1-13.
5. Canli T, Lesch KP. 2007. Long story short: the serotonin transporter in emotion regulation and social cognition. Nature Neuroscience 10(9): 1103-1109.
6. Cho S-B, Heron J, Aliev F, Salvatore J.E., Lewis G, Macleod J, Hickman M, Maughan B, Kendler K.S., Dick D.M. 2014. Alcoholism: clinical and experimental research. 38(7): 2024-2033.
7. Daws L.C., Montanez S, Munn J.L., Owens W.A., Baganz N.L., Boyce-Rustay J.M., Millstein R.A., Wiedholz L.M., Murphy D.L., Holmes A. 2006. Ethanol Inhibits Clearance of Brain Serotonin by a Serotonin Transporter-Independent Mechanism. Journal of Neurosience. 26 (24): 6431-6438.
8. Enoch M.A. 2011. The role of early life stress as a predictor for alcohol and drug dependence. Psychopharmacology. 214(1): 17-31
9. Farrell M.R., Holland F.H., Shansky R.M., Brenhouse H.C. 2016. Sex-specific effects of early life stress on social interaction and prefrontal cortex dendritic morphology in young rats. Behavioural Brain Research. 310:199-125.
10. Ford M.M., Eldridge J.C., Samson H.H. 2004. Determination of an estradiol dose-response relationship in the modulation of ethanol intake. Alcohol Clin Exp Res .28:20–28.
11. Gianchino C, Canalia N, Capone F, Fasolo A, Alleva E, Riva M.A., Circulli F, Peretto P. 2007. Maternal deprivation and early handling affect density of calcium binding protein-containing neurons in selected brain regions and emotional behavior in periadolescent rats. Neuroscience 145(2): 568-578.
12. Gorwood P, Batel P, Ades J, Hamon M, Boni C. 2000. Serotonin Transporter Gene Polymorphisms, Alcoholism, and Suicidal Behavior. Biological Psychiatry. 48 (4): 259-264
13. Grant B.F, Goldstein R.B, Saha T.D,Chou S.P, Jung J, Zhang H, Pickering R.P, Ruan W.J, Smith S.M, Huang B, Hasin D.S. 2015. Epidemiology of DSM-5 Alcohol Use Disorder: Results From the National Epidemiologic Survey on Alcohol and Related Conditions III. JAMA Psychiatry. 72(8):757-66.
14. Green J.G., McLaughlin K.A., Berglund P.A., Gruber M.J., Sampson N.A., Zaslavsky A.M., Kessler R.C. 2010. Childhood adversities and adult psychiatric disorders in the national comorbidity survey replication I: associations with first onset of DSM-IV disorders. Arch Gen Psychiatry 67:113–123
15. Gunnar M.R., Donzella B. 2002. Social regulation of the cortisol levels in early human development. Psychoneuroendocrinology. 27:199–220.
16. Heinz A, Ragan P, Jones D.W., Hornmet D, Williams W, Knable M.B., Gorey J.G., Doty L, Geyer C, Lee K.S. 1998. Reduced central serotonin transporters in alcoholism. American journal of psychiatry. 11: 1544-1549.
17. Holland F.H., Ganguly P, Potter D.N., Chartoff E.H., Brenhouse H.C. 2014. Early life stress disrupts social behavior and prefrontal cortexparvalbumin interneurons at an earlier time-point in femalesthan in males. Neuroscience Letters. 566:131-136.

18. Holmes A, Murphy D.L., Crawley J.N. 2002. Reduced aggression in mice lacking the serotonin transporter. Psychopharmacology (Berl.), 161: 160-167.
19. Homberg J.R., Pattij T., Janssen M.C., Ronken E., de Boer S.F., Schoffelmeer A.N., Cuppen E. 2007. Serotonin transporter deficiency in rats improves inhibitory control but not behavioural flexibility. Eur. J. Neurosci 26: 2066–2073.
20. Homberg J.R., Schiepers O.J.G., Schoffelmeer A.N.M., Cuppen E, Van Der Schuren L.J.M.J. 2008a. Acute and constitutive increases in central serotonin levels reduce social play behaviour in peri-adolescent rats. Psychopharmacology 195(2): 175.
21. Homberg J.R., de Boer S.F., Raaso H.S., Oliver J.D.A., Verheul M, Ronken E, Cools A.R., Ellenbroek B.A., Schoffelmeer A.N.M., van der Schuren L.J.M.J., de Vries T.J., Cuppen E. 2008b. Adaptations in pre- and postsynaptic 5-HT1a receptor function and cocaine supersensitivity in serotonin transporter knockout rats. Psychopharmacology. 200: 367-380.
22. Houwing D.J., Buwalda B, van der Zee E, de Boer S.F., Olivier J.D.A. 2017. The serotonin transporter and early life stress: Translational perspectives. Frontiers in cellular neuroscience. 11.
23. Kaidanovich-Beilin O, Lipina T, Vukobradovic I, Roder J, Woodgett J.R. 2011. Assessment of social interaction behaviors. J Vis Exp. 48: 2473.
24. Kaleuff A.V., Fox M.A., Gallagher P.S., Murphy D.L. 2007. Hypolocomotion, anxiety and serotonin syndrome-like behavior contribute to the complex phenotype of serotonin transporter knockout mice. Genes, Brain and Behavior 6:389-400.
25. Kaleuff A.V., Olivier J.D.A., Nonkes L.J.P., Homberg J.R. 2010. Conserved role for the serotonin transporter gene in rat and mouse neurobehavioral endophenotypes. Neuroscience and Biobehavioral rewies 34: 373-386.
26. Kelai S. 2003. Alcohol intake after serotonin transporter inactivation in mice. Alcohol and Alcoholism.
27. Kaplow J.B. and Widom C.S. 2007. Age of onset of child maltreatment predicts long-term mental health outcomes. J Abnorm Psychol. 116:176–187
28. Koss M.P., Yuan N.P., Dightman D, Prince R.J., Polacca M, Sanderson B, Goldman D. 2003. Adverse childhood exposures and alcohol dependence among seven native American tribes. Am J Prev Med. 25(3):238-244.
29. Lamb R.J, Daws L.C. 2013. Ethanol self-administration in serotonin transporter knockout mice: unconstrained demand and elasticity. Genes Brain Behav. 12(7): 741-7.
30. Lamb R.J., Pinkston J.W., Daws L.C. 2014. Ethanol Effects on Multiple Fixed Interval, Fixed Ratio Responding in Mice with Deletions of the Serotonin Transporter Gene. Behav Pharmacol. 25(1): 92-95.
31. Lesch KP. 2007. Linking emotion to the social brain: the role of the serotonin transport in human social behaviour. EMBO reports 8(S1): S24-S29.
32. Lewejohann L, Kloke V, Heiming R.S., Jansen F, Kaiser S, Schimitt A, Lesch K.P, Sacher N. 2010. Social status and day-to-day behaviour of male serotonin transporter knockout mice. Behavioural Brain Research 211:220-228.
33. Li Q, Wichems C, Heils A, van de Kar L.D., Lesch K.P., Murphy D.L. 1999. Reduction of 5-hydroxytryptamine (5-HT)(1A)-mediated temperature and neuroendocrine responses and 5-HT(1A) binding sites in 5-HT transporter knockout mice. J Pharmacol Exp Ther. 291(3): 999-1007.
34. Loi M, Mossink J.C., Meerhoff G.F., Den Blaauwen J.L., Lucassen P.J., Joels M. 2017. Effects of early-life stress on cognitive function and hippocampal structure in female rodents. Neuroscience, 342, 101–119.
35. Lovinger D.M. 1997. Serotonin's role in alochol's effects on the brain. Alcohol health & research wold 21(2):114-120.

36. Mancinelli R, Vitali M, Ceccanti M. 2009. Women, alcohol and the environment: an update and perspectives in neuroscience. Funct Neurol. 24:77–81.

37. Mcgue, M. The behavioral genetics of alcoholism. Current Directions in Psychological Science. 8:109– 115, 1999

38. Moy S.S., Nadler J.J., Young N.B., Nonneman R.J., Grossman A.W., Murphy D.L., D'Ercole A.J., Crawley J.N., Magnuson T.R., Lauder J.M. 2009. Social approach in genetically-engineered mouse lines relevant to autism. Genes Brain Behav. 8: 129–142.

39. Munafò M. R., Durrant C., Lewis G., Flint J. 2009. Gene × environment interactions at the serotonin transporter locus. Biol. Psychiatry 65, 211–219.

40. Nadler J.J, Moy S.S, Dold G, Trang D, Simmons N, Perez A, Young N.B, Barbaro R.P, Piven J, Magnuson T.R, Crawley J.N. 2004. Automated apparatus for quantitation of social approach behaviors in mice. Genes Brain Behav. 3(5): 303-14.

41. Murphy D.L, Andrews A.M, Wichems C.H, Li Q, Tohda M, Greenberg B. 1998. Brain serotonin neurotransmission: an overview and update with an emphasis on serotonin subsystem heterogeneity, multiple receptors, interactions with other neurotransmitter systems, and consequent implications for understanding the actions of serotonergic drugs. J Clin Psychiatry 59:4–12.

42. Nordquist N, Oreland L. 2010. Serotonin, genetic variability, behaviour, and psychiatric disorders--a review. Ups J Med Sci. 115(1):2-10.

43. Oliver J.D.A., Van Der Hart M.G.C., Van Swelm R.P.L., Dederen P.J., Homberg J.R., Cremers T, Deen P.M.T., Cuppen E, Cools A.R., Ellenbroek B.A. 2008. A study in male and female 5-HT transporter knockout rats: An animal model for anxiety and depression disorders. Neuroscience 152(3): 573-584.

44. Oo K.Z, Aung Y.K, Jenkins M.A, Win A.K. 2016. Associations of 5HTTLPR polymorphism with major depressive disorder and alcohol dependence: A systematic review and meta-analysis. Aust N Z J Psychiatry. pii: 0004867416637920

45. Page D.T., Kuti O.J., Prestia C, Sur M. 2009. Haploinsufficiency for Pten and serotonin transporter cooperatively influences brain size and social behavior. Proceedings-national academy of sciences USA 106(6): 1989-1994.

46. Palm S, Daoura L, Roman E, Nylander I, Homberg J. 2013. Effects of Rearing Conditions on Behaviour and Endogenous Opioids in Rats with Alcohol Access during Adolescence. PLoS ONE 8(10): e76591.

47. Reilly M.P, Weeks C.D, Topper V.Y, Thompson L.M, Crews D, Gore A.C. 2015. The effects of prenatal PCBs on adult social behavior in rats. Horm Behav. 73:47-55.

48. Roman E, Ploj K, Nylander I. 2004. Maternal separation has no effect on voluntary ethanol intake in female Wister rats. Alcohol. 33(1): 31-9.

49. Schubert D.S, Wolf A.W, Patterson M.B, Grande T.P, Pendleton L. 1988. A statistical evaluation of the literature regarding the associations among alcoholism, drug abuse, and antisocial personality disorder. Int J Addict. 23(8):797-808.

50. Schepis T.S, Rao U, Yadav H, Adinoff B. 2011. The limbic-hypothalamic-pituitary-adrenal axis and the development of alcohol use disorders in youth. Alcoholism: clinical and experimental research 35(4):595-605.

51. Sharpley C. F., Palanisamy S. K., Glyde N. S., Dillingham P. W., Agnew L. L. 2014. An update on the interaction between the serotonin transporter promoter variant (5-HTTLPR), stress and depression, plus an exploration of non-confirming findings. Behav. Brain Res. 273, 89–105.

52. Spanagel R. 2009. Alcoholism: a systems approach from molecular physiology to addictive behavior. Physiol Rev. 89(2):649-705.

20

53. Torres O.V., Walker E.M., Beas B.S., O'Dell L.E. 2014. Female rats display enhanced rewarding effects of ethanol that are hormone dependent. Alcoholism: Clinical and Experimental research. 38(1): 108-115.

54. Tuithof M, Ten Have M, van den Brink W, Vollebergh W, de Graaf R. 2013. Predicting persistency of DSM-5 alcohol use disorder and examining drinking patterns of recently remitted individuals: a prospective general population study. Addiction. 108(12):2091-9.

55. Van den Hove D.L., Jakob S.B., Schraut K.G., Kenis G, Schmitt A.G., Kneitz S, Scholz C.J., Wiescholleck V, Ortega G, Prickaerts J, Steinbusch H, Lesch K.P. 2011. Differential effects of prenatal stress in 5-Htt deficient mice: towards molecular mechanisms of gene × environment interactions. PloS one. 6(8): e22715.

56. Verheij M.M.M., Karel P, Cools A.R., Homberg J.R. 2014. Reduced cocaine-induced serotonin, but not dopamine and noradrenaline, release in rats with a genetic deletion of serotonin transporters. European Neuropsychopharmacology. 24 (11): 1850-1854.

57. Vonghia L, Michelsen P, Dom G, Fancque S. 2014. Diagnostic challenges in alcohol use disorder and alcoholic liver disease. World J Gastroenterol. 20(25):8024-32.

58. Walker C-D, Bath K.G., Joels M, Korosi A, Larauche M, Lucassen P.J., Morris M.J., Raineki C, Roth T.L., Sullivan F, Taché Y, Baram T.Z. 2017. Chronic early life stress induced by limited bedding and nesting (LBN) material in rodents: critical considerations of methodology, outcomes and translational potential. Stress. 20(5):421-448.

59. Watson K.K, Ghodasra J.H., Platt M.L. 2009. Serotonin transporter genotype modulates social reward and punishment in rhesus macaques. PloS one 4(1):e4156.

60. Young R, Sweeting H, West P. 2007. A longitudinal study of alcohol use and antisocial behaviour in young people. Alcohol & Alcoholism. 43(2): 204-214.