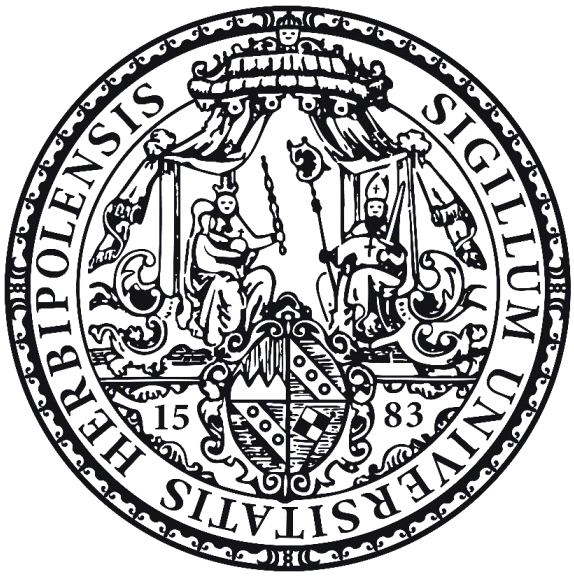

Fear and anxiety disorders – interaction of AVP and OXT brain systems with the serotonergic system

Furcht und Angsterkrankungen – Interaktion von AVP und OXT Gehirnsystemen mit dem serotonergen System

Doctoral thesis for a doctoral degree
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Abbreviations

°C	Degree Celsius
µm	Micrometer
5-HT	5-Hydroxytrytamin, Serotonin
5-HTT	Serotonin transporter
5-HTTLPR	Serotonin-transporter-linked promoter region
a	Anterior
ACC	Anterior cingulate cortex
Actb/ACTB	Actin beta
ACTH	Adrenocorticotropic hormone
ADHD	Attention deficit hyperactivity disorder
Amy	Amygdala
ANOVA	Analysis of variance
Arc/ARC	Activity-regulated cytoskeleton-associated protein
Avp/AVP	Arginin-Vasopressin
Avpr1a/AVPR1A	Vasopressin receptor 1a
B2m/B2M	Beta 2 microglobulin
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
BNST	Bed nucleus of the stria terminalis
bp	Base pair
BSA	Bovine serum albumin
C	Control
CA	Cornu ammonis
CA1/2/3	Cornu ammonis area 1/2/3
CBT	Cognitive behavioral therapy
cDNA	Complementary DNA
cFos/cFOS	Fos proto-oncogene
CNS	Central nervous system
CORT	Corticosterone
CRH	Corticotropine releasing hormone
Crhr1/CRHR1	Corticotropine releasing hormone receptor 1
CSF	Cerebrospinal fluid
Ct	Threshold cycle
d	Dorsal

DA	Dopamine
DAB	3,3`-diaminobenzidin
DEPC	Diethyl pyrocarbonate
DG	Dentate gyrus
DLS	Dorsolateral septum
DNA	Deoxyribonucleic acid
dNTP	Desoxynucleoside triphosphate
DR	Dorsal raphe
DSM-V	Diagnostic and Statistical Manual of Mental Disorders, fifth edition
E	Embryonic day
EDTA	Ethylenediaminetetraacetic acid
EPM	Elevated plus maze
EtOH	Ethanol
Fosl2/FOSL2	Fos-related antigen 2
GABA	Gamma-Aminobutyric acid
GAD	Generalized anxiety disorder
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GCL	Granular cell layer
Gdi2/GDI2	guanosine diphosphate dissociation inhibitor 2
GWAS	Genome wide association study
GxE	Gene-environment interaction
hi	Hilus
h	Hour(s)
H_2O_2	Hydrogen peroxide
HAM-A	Hamilton Anxiety Rating Scale
Het	Heterozygous
HPA-axis	Hypothalamic-pituitary-adrenal axis
Htr1a/2a/2c / HTR1A/2A/2C	Serotonin receptor 1a/2a/2c
IEG	Immediate early gene
IF	Immunofluorescence
IgG	Immunoglobulin G
ir	Immunoreactive
KO	Knockout
LMD	Laser microdissection

mA	Milliampere
MAO-A	Monoamine oxidase A
min	Minute(s)
miRNA	Micro ribonucleic acid
Mo	Molecular layer
mPFC	Medial prefrontal cortex
mRNA	Messenger ribonucleic acid
n	Sample size
NA	Noradrenaline
NA	Numerical aperture
NAcc	Nucleus accumbens
NGS	Normal goat serum
NHS	Normal horse serum
nm	Nanometer
NPSR1	Neuropeptide S receptor
Npy/NPY	Neuropeptide Y
Npyr1/2 | NPYR1/2	Neuropeptide Y receptor type 1/2
OCD	Obsessive-compulsive disorder
OD	Optical density
OFT	Open field test
Oxt/OXT	Oxytocin
Oxtr/OXTR	Oxytocin Receptor
p	Posterior
p	P-value
PBS	Phosphate buffered saline
PCL	Pyramidal cell layer
PCR	Polymerase chain reaction
PEN	Polyethylene-naphthalate
PFA	Paraformaldehyde
PS	Prenatal stress
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus
qPCR	Real-time quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
Rplp0/RPLP0	60S acidic ribosomal protein P0
rpm	Revolutions per minute
RT	Room temperature

RT	Reverse Transcriptase
s	short
SAD	Social anxiety disorder
SCN	Suprachiasmatic nucleus
SDS	Sodium dodecyl sulfate
sec	Second
SEM	Standard error of mean
SFC	Social Fear Conditioning
SNP	Single-nucleotide polymorphism
SNRI	Selective norepinephrine reuptake inhibitor
so	Stratum oriens
SON	Supraoptic nucleus
sr	Stratum radiatum
SSRI	Selective serotonin reuptake inhibitor
TBS	Tris buffered saline
TMEM132D	Transmembrane receptor 132D
TPH	Tryptophan hyroxylase
Tph1/2 | TPH1/2	Tryptophan hydroxylase 1/2
TRIS	Tris (hydroxymethyl)-aminomethane
V	Voltage
WT	Wild type

Zusammenfassung

Angsterkrankungen sind eine große Belastung für Gesellschaft und Wirtschaft und können verheerende Folgen für Betroffene haben. Behandlungsmöglichkeiten sind nach wie vor auf Psychopharmakotherapie, welche ursprünglich für die Behandlung von Depressionen entwickelt wurde, und Verhaltenstherapie beschränkt. Eine Kombination aus bestimmten genetischen Eigenschaften zusammen mit aversiven Lebensereignissen sind die wahrscheinlichste Ursache für die Entstehung dieser Erkrankungen. Gen x Umweltstudien versuchen dabei, Verbindungen zwischen genetischen Merkmalen und spezifischen negativen Ereignissen zu finden. In einer ersten Studie haben wir uns auf die soziale Phobie konzentriert, welche die zweithäufigste Angsterkrankung nach spezifischen Phobien ist. Wir haben ein soziales Furchtkonditionierungs-Paradigma (*social fear conditioning*, SFC), verwendet, welches in der Lage ist, die soziale Phobie im Tiermodell nachzustellen. Wir haben nach einer Verbindung zwischen dem serotonergen System und den zwei Systemen der Neuromodulatoren Vasopressin (AVP) und Oxytocin (OXT) gesucht. Diese Neuropeptide beeinflussen im Gehirn als Neuromodulatoren das Verhalten, und regulieren sowohl positive als auch negative Aspekte des Sozial- und Angstverhaltens. Eine gegenseitige Beeinflussung dieser Neurotransmittersysteme bei der Entstehung von Angsterkrankungen zu identifizieren könnte dabei helfen, potentielle Ziele für neue Behandlungsansätze zu finden. Diese werden dringend benötigt, da der prozentuale Anteil der Patienten, für die es keine wirksame Behandlung gibt, hoch ist.
Wir haben Proteinebene und mRNA Expression von unmittelbar frühen Genen (immediate early genes, IEGs) analysiert, um zu ermitteln, in welchen Hirnregionen die neuronale Aktivität durch das Paradigma beeinflusst wird. Außerdem wurde in dieser Studie eine Untersuchung der Gene von AVP-, OXT-, Neuropeptid Y (NPY)-Systemen, sowie von Genen des serotonergen Transmissionssystems eingeschlossen. Damit sollten die Auswirkungen von SFC auf die Genexpression in Hirnregionen, die mit Sozial- sowie Angstverhalten in Verbindung stehen, ermittelt werden.
Wir konnten sowohl eine veränderte Genexpression von verschiedenen IEGs wie cFos und Fosl2, als auch Veränderungen in Zahl und Dichte von cFOS-positiven Zellen feststellen, was einen Einfluss von SFC auf neuronale Aktivität andeutet. Unsere Ergebnisse offenbaren eine mögliche Beteiligung des Gyrus dentatus (DG), sowie der Cornu ammonis area 1 (CA1) und CA3 im dorsalen Hippocampus bei der Expression von sozialer Angst. Entgegen unseren Vermutungen waren in der Amygdala keine Veränderungen der neuronalen Aktivität durch Expressionsänderungen der IEGs nachzuweisen. Signifikant höhere IEG-Immunreaktivität und -Genexpression im dorsalen Hippocampus von Tieren ohne Furchtkonditionierung (SFC-) im Vergleich zu Tieren mit Furchtkonditionierung (SFC+) weisen auf zwei mögliche Szenarien hin. Entweder handelt es sich um eine verstärkte Expression in SFC--Tieren im Vergleich zu SFC+-Tieren, oder die Expression in SFC+-Tieren ist im Vergleich zu SFC--Tieren erniedrigt. Ohne eine zusätzliche Kontrolle der basalen mRNA Konzentration und des Proteinvorkommens der IEGs in einer Kontrollgruppe ohne soziale Interaktionsmöglichkeit kann diese Frage allerdings nicht beantwortet werden. Das NPY-System generell und der NPY-Rezeptor 2 im Speziellen scheinen in die Regulation der Reaktion auf soziale Angst involviert zu sein,

und dies hauptsächlich im Septum. Zusätzlich konnte eine mögliche Rolle für das serotonerge System und insbesondere den Serotonin Rezeptor 2a im Nucleus paraventricularis (PVN) bei der Reaktion auf soziale Angst identifiziert werden.

In einer zweiten Studie haben wir uns auf Veränderungen des serotonergen Systems konzentriert. Ein Polymorphismus im humanen Serotonintransporter Gen (*5-HTT*) konnte mit einem höheren Risiko für Angsterkrankungen assoziiert werden. Dies macht den 5-HTT zu einem weit verbreiteten Ziel zur Erforschung von möglichen Ursachen und der Entwicklung von Angsterkrankungen. In Mäusen ist ein gentechnisch induzierter knockout des *5-Htt* Gens mit erhöhtem Angstverhalten assoziiert. Ein hohes Stresslevel während der Schwangerschaft, auch als pränataler Stress bekannt, erhöht das Risiko für spätere psychiatrische Erkrankungen des noch ungeborenen Kindes signifikant. In unserer Studie haben wir ein pränatales Stress-Paradigma in Mäusen mit einer Defizienz des *5-Htt* Gens verwendet. In einer vorangegangenen Studie hatten sich bereits einige der Tiere, die pränatalem Stress ausgesetzt waren, in der Interaktion mit anderen Tieren auffällig „unsozial" verhalten, bzw. geringes Sozialverhalten gezeigt. Wir haben erneut mithilfe von Genexpressionsstudien nach einer Verbindung zwischen dem serotonergen System und den AVP- und OXT-Systemen gesucht. Zusätzlich haben wir AVP und OXT in Mäusen mit verschiedenen *5-Htt* Genotypen und in beiden Geschlechtern auf Neuropeptidebene analysiert, um zu sehen, ob die Produktion von AVP und OXT durch den *5-Htt* Genotyp und das Geschlecht beeinflusst ist.
Im Zuge der quantitativen Genexpressionsstudie konnten wir zeigen, dass die AVP- und OXT- Neuropeptidsysteme in weiblichen, aber nicht in männlichen Mäusen, durch Pränatalstress beeinflusst werden. Der *5-Htt* Genotyp scheint AVP, OXT und andere untersuchte Neurotransmittersysteme nur geringfügig zu beeinflussen. In Weibchen ist die Genexpression von *Oxt* und *Oxtr* teilweise stark durch den Östruszyklus beeinflusst. Auf Neuropeptidebene konnten wir einen Geschlechterunterschied bzgl. der durchschnittlichen Anzahl AVP-positiver Zellen im PVN feststellen; männliche Tiere hatten signifikant mehr positive Zellen als weibliche Tiere.

Summary

Anxiety disorders pose a great burden onto society and economy and can have devastating consequences for affected individuals. Treatment options are still limited to psychopharmacotherapy originally developed for the treatment of depression and behavioral therapy. A combination of genetic traits together with aversive events is most likely the cause of these diseases. Gene x environment studies are trying to find a link between genetic traits and specific negative circumstances. In a first study, we focused on social anxiety disorder (SAD), which is the second most-common anxiety disorder after specific phobias. We used a social fear conditioning (SFC) paradigm, which is able to mimic the disease in a mouse model. We wanted to investigate protein levels, as well as mRNA expression of immediate early genes (IEGs), to determine brain areas affected by the paradigm. We also included genes of the vasopressin (AVP)-, oxytocin (OXT)-, neuropeptide Y (NPY)-, and the serotonin system, to investigate the effects of SFC on neurotransmitter gene expression levels in brain regions related to social as well as fear-related behavior. AVP and OXT regulate a lot of different social and anxiety-related behaviors, both positive and negative. Finding a link between different neurotransmitter systems in the development of anxiety disorders could help to identify potential targets for new treatment approaches, which are desperately needed, because the rate of patients not responding to available treatment is very high.

We were able to show altered gene expression of the IEGs cFos and Fosl2, as well as a change in number and density of cFOS-positive cells in the dorsal hippocampus, indicating an influence of SFC on neuronal activity. Our results reveal a possible involvement of anterior dentate gyrus (DG), as well as cornu ammonis area 1 (CA1) and CA3 in the dorsal hippocampus during the expression of social fear. Contrary to our hypothesis, we were not able to see changes in neuronal activity through expression changes of IEGs in the amygdala. Significant higher IEG immunoreactivity and gene expression in the dorsal hippocampus of animals without fear conditioning (SFC-), compared to animals with fear conditioning (SFC+), indicate an involvement of different hippocampal regions in two possible scenarios. Either as elevated gene expression in SFC- animals compared to SFC+ animals or as reduction in SFC+ animals compared to SFC- animals. However, this question cannot be answered without an additional control of basal IEG-activity without social interaction. The NPY system in general and the neuropeptide y receptor type 2 in particular seem to be involved in regulating the response to social fear, mostly through the septum region. In addition to that, a possible role for the induction of social fear response could be identified in the serotonergic system and especially the serotonin receptor 2a of the PVN.

In a second study we focused on changes in the serotonergic system. A polymorphism in the human serotonin transporter (5-HTT) gene is associated with higher risks for the development of anxiety disorders. This makes the 5-HTT a widely used target to study possible causes and the development of anxiety disorders. In mice, a genetically induced knockout of the *5-Htt* gene is associated with increased anxiety-like behavior. High amounts of stress during pregnancy, also known as prenatal stress, significantly increase

the risk to develop psychiatric disorders for the unborn child. We utilized a prenatal stress paradigm in mice heterozygous for the *5-Htt* gene. Some of the animals which had been subjected to prenatal stress showed noticeably "unsocial" interaction behavior towards conspecifics. Again, we were searching for links between the serotonergic system and AVP- and OXT systems. Through quantitative gene expression analysis, we were able to show that both AVP and OXT neuromodulator systems are affected through prenatal stress in female mice, but not in male mice. The *5-Htt* genotype seems to be only slightly influential to AVP, OXT or any other neurotransmitter system investigated. Gene expression of AVP and OXT brain systems is highly influenced through the estrous cycle stages of female mice. Additionally, we analyzed the AVP and OXT neuropeptide levels of mice with different *5-Htt* genotypes and in both sexes, in order to see whether the production of AVP and OXT is influenced by *5-Htt* genotype. On neuropeptide level, we were able to identify a sex difference for vasopressin-immunoreactive (ir) cells in the PVN, with male mice harboring significantly more positive cells than female mice.

1 Introduction

1.1 Anxiety disorders and risk factors

Among the spectrum of psychiatric disorders, anxiety disorders represent the highest percentage (Penninx et al. 2021). They are subdivided into panic disorder and agoraphobia, which mostly have their onset in adulthood; social anxiety disorder (SAD), generalized anxiety disorder (GAD) and specific phobias with onset points between childhood and adulthood; as well as selective mutism and separation anxiety which occur during childhood (figure 1). Until the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V), obsessive-compulsive disorders (OCD) and trauma-related disorders like post-traumatic stress disorder (PTSD) were also part of the anxiety disorder group but have since received own categories.

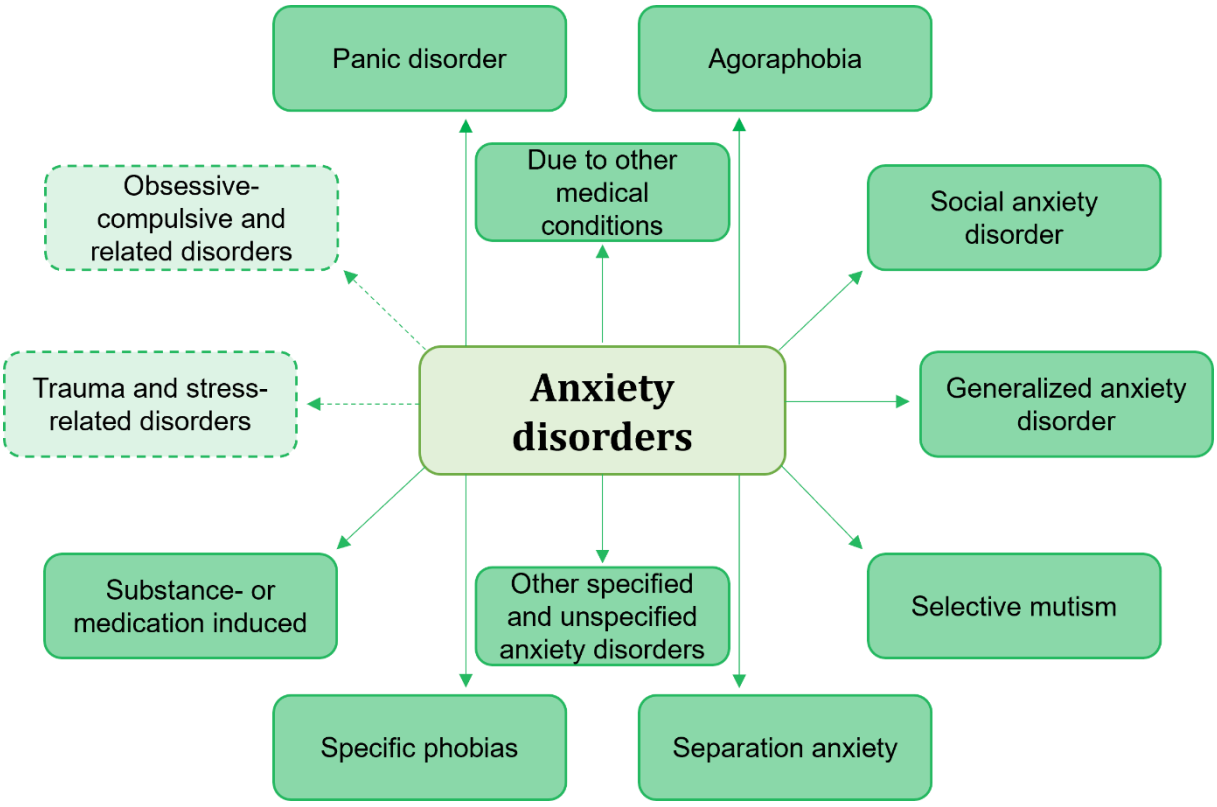

Figure 1: Visual representation of the different anxiety disorder categories.
Visual representation of the different anxiety disorder categories. Obsessive-compulsive disorders (OCD) and post-traumatic stress disorder (PTSD) were previously listed under anxiety disorders but received their own category according to DSM-V. Adapted from (Craske and Stein 2016; Craske et al. 2017).

A combination of high prevalence together with debilitating effects caused by chronic disease progress and many comorbidities lead to anxiety disorders being ranked as the ninth-most cause of disability in the context of health (Vos et al. 2017). The impact on society and economics is immense. More than 50% of the patients that are diagnosed with an anxiety disorder fulfill the criteria of at least one other anxiety disorder, which makes the disease spectrum highly comorbid (Lamers et al. 2011). Robust prevalence rates are

difficult to determine. Two studies looking at all anxiety disorders in an age range between adolescence and early adulthood reported cumulative prevalence rates of 20% to 30% (Copeland et al. 2014; Ormel et al. 2015). From an evolutionary perspective, fear and anxiety are both important to evaluate threats and dangers. Fear as an emotion can be perceived during dangerous and potentially life-threatening situations and is a normal reaction that helps to drive the survival instinct. Anxiety is described as the anticipation of stressful, dangerous or threatening scenarios in the future, which can pose an actual threat or be imaginative (Penninx et al. 2021). The source of these emotions are usually day-to day situations in which the sensation of fear is considered to be a normal reaction. However, the perception of fear and anxiety can become immensely excessive in comparison to the actual threat, which is one of the core symptoms of any anxiety disorder. In general, the characteristics of anxiety disorders are very similar, with a source of the fear leading to "marked, excessive and unreasonable fear or anxiety" (*Diagnostic and statistical manual of mental disorders : DSM-5* 2013). Compared to other psychiatric disorders, the onset of anxiety disorders is noticeably earlier, with diseases like separation anxiety and selective mutism being very prominent in small children (Solmi et al. 2022; Bandelow and Michaelis 2015). Anxiety disorders are in general both underdiagnosed and undertreated (Alonso et al. 2018).

Diagnosis and treatment observation are usually supported through interviews with the patient, utilizing standardized questionnaires like the Hamilton Anxiety Rating Scale (HAM-A) (Zigmond and Snaith 1983). State of the art treatment options include behavioral therapies, with cognitive behavioral therapy (CBT) having the strongest evidence among the spectrum of evidence-based psychotherapeutic options (Penninx et al. 2021). CBT based approaches usually consist of therapeutic sessions where the patient is confronted with the source of their fear, so-called exposure sessions. The individual learns through exposure to either real fearful stimuli, usage of virtual reality or their own imagination, that the feared consequences do not arise. The ideal outcome leads to the extinction of avoidance behavior in relation to the fearful stimulus. The second pillar of treatment is comprised of pharmacotherapy, which comes into play either as a component together with CBT, or in cases of treatment resistance to psychotherapy. Different classes of selective serotonin reuptake inhibitors (SSRIs) and selective norepinephrine reuptake inhibitors (SNRIs) deliver mostly moderate effects in several anxiety disorders (Bighelli et al. 2018; Curtiss et al. 2017; Gomez, Barthel, and Hofmann 2018). For the acute treatment of states of anxiety or panic attacks, benzodiazepines can be used, as they have relaxing and anxiolytic effects (Gomez, Barthel, and Hofmann 2018; Breilmann et al. 2019) However, due to the high potential of dependency, benzodiazepines cannot be used as a long-term treatment option. A combination of psychotherapy and pharmacotherapy as treatment options delivers better results than both treatment lines alone (Cuijpers et al. 2014). The amount of treatment resistant patients is still posing a high demand for the development of alternative treatment options and diagnostic and treatment evaluations, like the standardization of diagnostic procedures or monitoring of the treatment outcome (Penninx et al. 2021). Further

promising drug classes target the glutamatergic system (Taylor et al. 2018; Glue et al. 2020), the endocannabinoid system or corticotropin-releasing hormone receptors (Penninx et al. 2021).

Looking for risk factors facilitating anxiety disorders, a combination of genetic and epigenetic factors becomes apparent. Compared to other psychiatric disorders like schizophrenia and autism, heritability rates of anxiety orders are relatively low, ranging between 35% and 50%, depending on the specific disorder (Meier and Deckert 2019). Even though single contributing genetic factors have not been found, several risk genes are implied to have an effect on the development of anxiety disorders. Risk genes associated with panic disorder are transmembrane receptor 132D (*TMEM132D*), *HTR2A*, neuropeptide S receptor (*NPSR1*) and *MAO-A* (Howe et al. 2016). Attempts to find genetic loci through genome wide association studies (GWAS) have not been successful yet. However, genetic correlations seem to be present in people affected by different types of psychiatric disorders (Meier et al. 2019; Purves et al. 2020; Forstner et al. 2021). Epigenetic modifications of DNA involve alterations on DNA level, which do not change the overall DNA sequence, but lead to functional changes. One of the most common epigenetic mechanisms is methylation, where methyl groups are added to the DNA. This leads to alterations in the activity of the DNA segment. Methylation patterns in humans can be altered through epigenetic influence. It is difficult to study methylation in human samples, because they can only be assessed within blood samples. Using the peripheral approach, *MAO-A* (Ziegler et al. 2016), Corticotropin-releasing hormone receptor 1 (*CRHR1*) (Schartner et al. 2017) and *OXTR* (Ziegler et al. 2015) were found to have differing methylation. Definite genetic markers for psychiatric disorders cannot be identified to this day, but the seeming genetic heritability between patients of different psychiatric disorders give a strong hint that a specific genetic preset combined with aversive elements trigger the onset of psychiatric disease (Penninx et al. 2021).

1.2 Social anxiety disorder

After specific phobias, social anxiety disorder is the second most common anxiety disorder with lifetime prevalence in the US of around 12% (Kessler et al. 2012) and yearly prevalence rates of around 2.3% (Wittchen and Jacobi 2005). The Diagnostic and Statistical Manual of Mental Disorders (DSM-V)) describes the core symptoms of SAD in its fifth edition as a profound fear or anxiety to be evaluated or possibly scrutinized by others during social situations, for instance having a conversation with unknown individuals or delivering a speech in front of an audience (Leichsenring and Leweke 2017). The characteristics and particular situations affected by SAD are very diverse (Stein, Torgrud, and Walker 2000; Ruscio et al. 2008). Manifestation of fearful behavior ranges from little restrictions during everyday life situations to complete withdrawal from all social situations (Craske and Stein 2016). This can be severely debilitating for their living conditions and sometimes even the ability to work (Stein and Stein 2008). In the past, SAD was classified into a generalized and a non-generalized form, where the fear was related to a specific type of situation. However, in recent years, the distinction was

found not to be advantageous (Bögels et al. 2010). As with many disorders of the anxiety-related spectrum, the onset is early and can already affect children, with a mean onset of 13 years and a high possibility of becoming chronic (Stein and Stein 2008; Steinert et al. 2013; Leichsenring and Leweke 2017). The most common comorbid disorders include other anxiety disorders, as well as depression and substance use disorders (Ruscio et al. 2008). Most patients are diagnosed with at least one other psychiatric disorder , which makes comorbid psychiatric disorders very common (Koyuncu et al. 2019), about a quarter of the patients are diagnosed with not less than three psychiatric diseases (Chartier, Walker, and Stein 2003). At the moment, no treatment options specifically designed for the treatment of SAD are available. Patients show the best response rates after treatment with SSRIs (Williams et al. 2017). Pharmacotherapy together with the utilization of cognitive behavioral therapy (CBT) are considered to be state of the art therapy for SAD patients (Pelissolo, Abou Kassm, and Delhay 2019). With no causal treatment available, the rate of non-responding patients to the available treatment options is still very high (Blanco et al. 2013; Williams et al. 2017; Pelissolo, Abou Kassm, and Delhay 2019), which highlights the demand for the development of alternative options.

Most likely, a combination of genetic and environmental factors drives the development of SAD and are responsible for intra-individual differences in the manifestation of symptoms (Scaini, Belotti, and Ogliari 2014). With a series of diverse factors influencing the emergence of SAD, the brain mechanisms and neural circuits involved remain mostly unclear, despite numerous attempts of uncovering them (Duval, Javanbakht, and Liberzon 2015). Functional studies regarding the involved brain regions revealed mixed and sometimes contradictory results. In SAD patients, volume of the amygdala, a region responsible for fear learning and expression (Fanselow and LeDoux 1999), was found reduced (Irle et al. 2010), enlarged (Machado-de-Sousa et al. 2014) or comparable (Syal et al. 2012) to control groups. However, activity of the amygdala region was mainly reported to be increased in SAD patients (Tillfors et al. 2001; Lorberbaum et al. 2004; Phan et al. 2006; Guyer et al. 2008). The hippocampus is responsible for general learning processes (Squire 1992) and in the context of fearful stimuli processing of the context of this situation (Kim, Rison, and Fanselow 1993). SAD is associated with morphological changes of the hippocampus. Both volume and density were found to be decreased in SAD patients compared to healthy controls (Irle et al. 2010; Liao et al. 2011; Qiu et al. 2011), and brain activity in this region seems to be altered (Kilts et al. 2006; Schneider et al. 1999).

1.2.1 Animal models for social anxiety disorder

The fact that it is not possible to mimic performance anxiety or the fear of being evaluated by others in animals shows the limitations of an animal model for SAD. However, some approaches for behavioral models have been able to concentrate on the social interaction part regarding the spectrum of SAD symptoms. Behavioral fear response directed towards a conspecific or its behavior can be interpreted as social fear (Toth and Neumann 2013).

Since mice are considered “social” animals, encountering an unknown conspecific normally triggers interest and interaction approaches. Many paradigms designed to assess social fear and interaction are based on the idea that a reduction in or retraction from social interaction translates to social fear. One of the oldest experimental approaches regarding social fear is the social interaction test. Two conspecifics of the same sex that are unfamiliar with each other are placed within the same unknown environment and the interaction is monitored (File and Hyde 1978). While the degree of social interaction between the two animals can be measured and decreased interaction is a sign of social avoidance, it is intricate to determine the amount of interaction behavior exhibited by each individual (Toth and Neumann 2013). To circumvent this issue and to be able to determine the level of social interaction individually, a range of similarly designed paradigms have been implemented. The joint similarity between the three-chamber sociability test (Landauer and Balster 1982), the social approach/avoidance test (Haller and Bakos 2002) and the social preference/avoidance test (Berton et al. 2006) is the fact that it allows the evaluation of a single animal and its reaction to an unknown conspecific. All these paradigms are designed to allow social interaction approaches only from one conspecific, whereas the second animal is used as a social cue and cannot initiate interaction by itself due to being in a separated compartment. The results gained allow to draw conclusions regarding social avoidance behavior in animal models that show enhanced fear and anxiety compared to control groups. More recent approaches are aimed at behavioral paradigms which specifically induce fear and avoidance in social situations and therefore mimic SAD in humans. The spectrum of these approaches includes different forms of physical and social stressors either alone or combined (Toth and Neumann 2013). The most used approaches use foot shocks or restraint stress as physical stressors since these methods are widely applied in behavioral paradigms and cause significant stress for the animals. Successful animal models not only have to be able to mimic the physiological and physical symptoms of SAD (Réus et al. 2014). It is also important that the treatment options used in human therapy are able to reverse alterations caused by the paradigm (Willner 1986). Social fear conditioning (SFC) is the first experimental paradigm for the specific induction of social fear in mice (Toth, Neumann, and Slattery 2012a). Experimental mice are subjected to an unknown conspecific in a wire mesh cage. Once the mice try to interact with a conspecific, they receive a foot shock and in consequence learn to associate their own voluntary social interaction behavior with negative consequences. This leads to withdrawal from social interaction approaches and fear of social situations. Other areas of behavior, like investigation behavior or general anxiety remain unchanged by the treatment, which makes SFC a solid tool for the investigation of SAD (Toth, Neumann, and Slattery 2013).

1.3 Brain neurotransmitter systems and social behavior

1.3.1 The serotonergic system

With about 95% (Gershon and Tack 2007), the vast majority of serotonin (5-HT) in the body is located outside of the central nervous system (CNS), more precisely in the gut. It functions as a hormone and regulates a wide range of physiological functions (Jones et al. 2020) including metabolism (Martin et al. 2017), functions of the gastrointestinal tract (Keating and Spencer 2019) and vasoconstriction (Rapport, Green, and Page 1948). The serotonergic system within the brain is one of the key neurotransmitter systems and has important functions regulating mood, anxiety and happiness (Lucki 1998). Serotonin belongs to the monoamine neurotransmitter family together with dopamine (DA), adrenaline and noradrenaline (NA). The production of serotonin is conducted in serotonergic neurons, mainly within the soma. The pathways of the serotonergic brain system are depicted in figure 2.

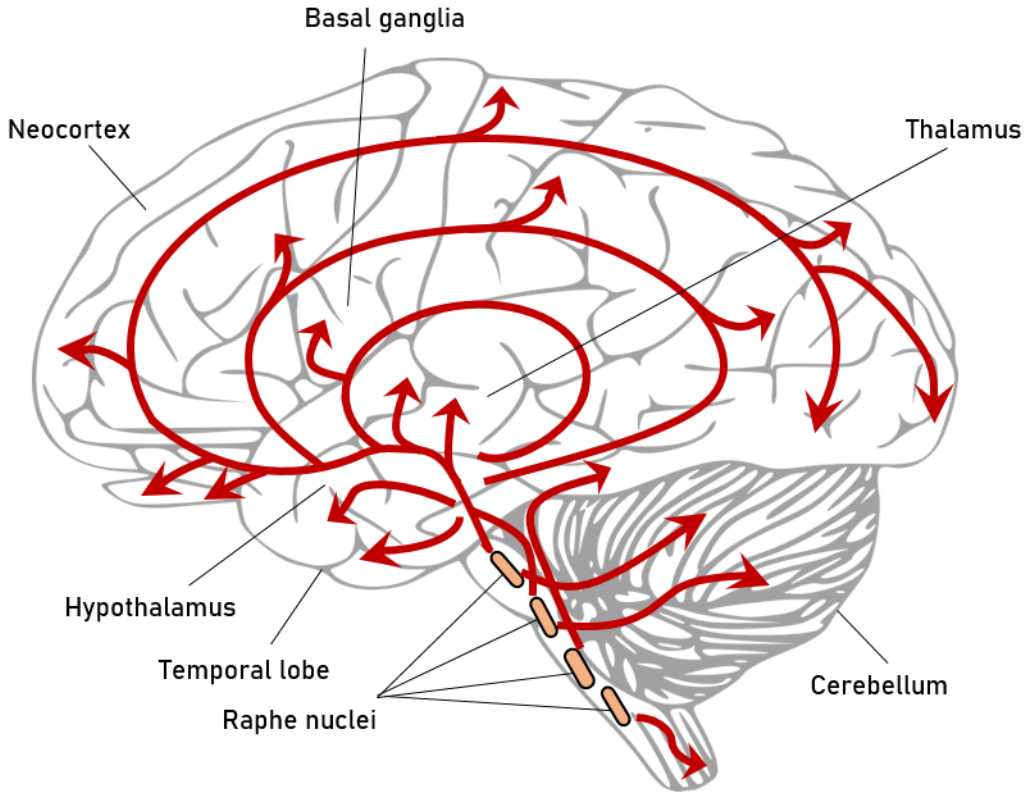

Figure 2: Pathways of the serotonergic brain system.
Adapted from (Bear, Connors, and Paradiso 2018), p. 582.

Serotonergic neurons are seated in the raphe nuclei, which are located as cell clusters in the brain stem. Based on the areas of projection, they can be divided into two different complexes. The caudal raphe complex is located in the medulla oblongata and caudal pons, whereas the rostral raphe complex is located in the midbrain and the rostral pons. Projections of the rostral complex reach into diencephalon and forebrain, where fibres project into the cortex, amygdala, hippocampus and striatum. (Kriegebaum et al. 2010).

The rate limiting enzyme for the production of serotonin is the tryptophan hydroxylase (TPH), which hydroxylizes L-tryptophane to 5-hydroxytryptohan. Serotonin synthesis in the brain is exclusively regulated through the TPH isoform TPH2, whereas both TPH2 and the other isoform, TPH1 are expressed in the periphery. Only TPH1 is expressed in the pineal gland (Gutknecht et al. 2009). Serotonin release happens from neurons mainly into the extracellular space, where it binds to serotonin-specific receptors. Seven different receptor families (5HTR1-7) are known to this day, with both inhibitory and excitatory capabilities, as well as different receptor types (Hoyer, Hannon, and Martin 2002). Serotonin reuptake into the presynaptic neuron is achieved through the serotonin transporter (5-HTT) (Zhou et al. 1998). Research into the function of 5-HTT delivered insights into its involvement in the development of anxiety-related traits. In humans, a length polymorphism in the *5-HTT* promotor is known to influence susceptibility to anxiety disorders and depression (Lesch et al. 1996). Two short (S) alleles of the serotonin-transporter-linked promoter region (*5-HTTLPR*), lead to significantly less mRNA and protein (figure 3). Individuals with two short alleles show higher levels of neuroticism, a trait associated with higher susceptibility to anxiety and depression (Greenberg et al. 2000). If tested for cognitive abilities, individuals with two S alleles perform better in tasks related to learning and decision making (Madsen et al. 2011; Borg et al. 2009; Roiser et al. 2007; Strobel et al. 2007).

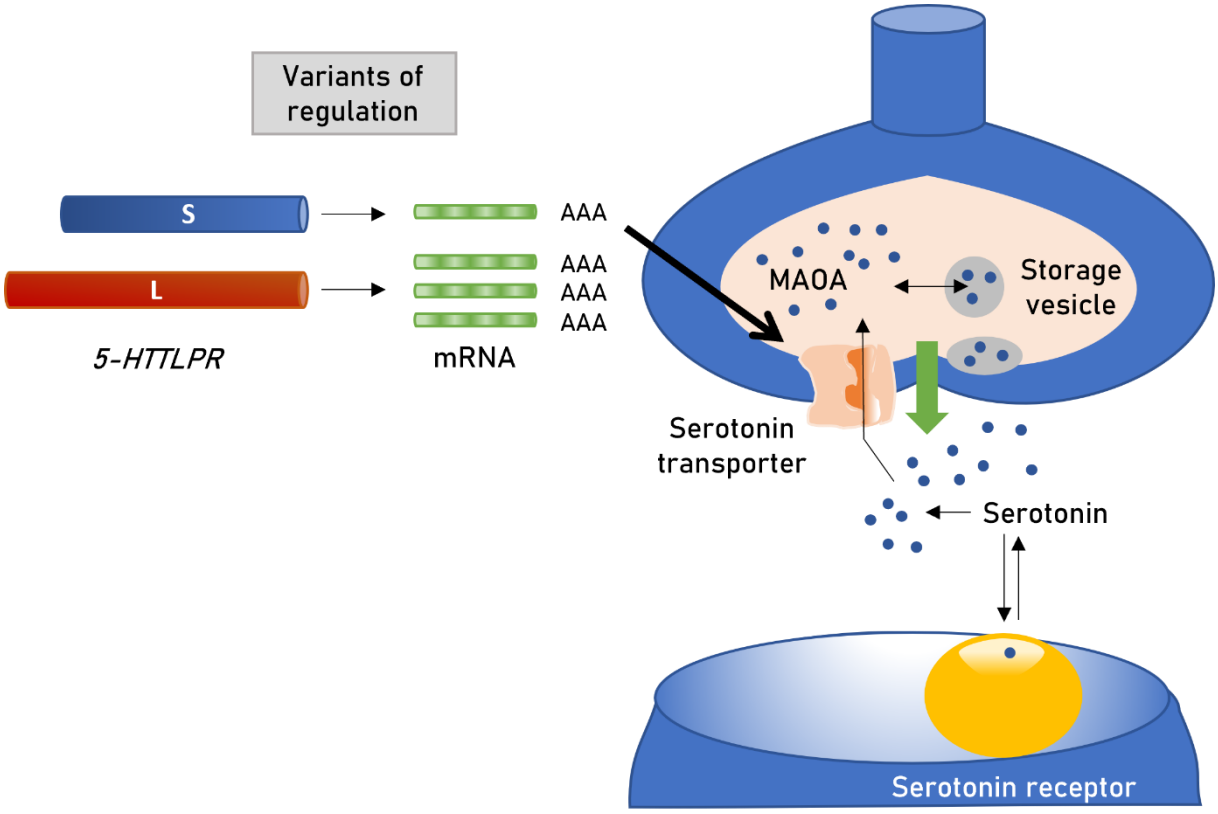

Figure 3: Polymorphism in the *5-HTT* gene promotor region leads to altered mRNA and protein levels.
Short (S) and long (L) *5-HTTLPR* variant of the *5-HTT* gene. The short variant leads to significantly less mRNA and protein and in the end higher concentrations of serotonin in the synaptic cleft. MAOA, monoamine oxidase A. Adapted from (Canli and Lesch 2007).

Genetic traits are usually not alone responsible for the development of psychiatric disorders, usually a combination of genetics and negative environmental influences (gene-environment interaction, GxE) trigger the emergence of the disease. Humans who carry two short alleles of the promotor gene variant are significantly more susceptible to the development of depression after the experience of stressful negative life events (Caspi et al. 2003; Kaufman et al. 2004; Kendler et al. 2005). While a complete 5-HTT knockout is possible, mice heterozygous for the *5-Htt* knockout show a phenotype more realistic and comparable to the situation of two short *5-HTTLPR* variants in humans.

1.3.2 5-HTT-KO mice as a model for anxiety disorders

Mice with reduced levels of 5-HTT exhibit elevated levels of anxiety-related/-like behavior and are an established model to investigate the development of anxiety disorders (Bengel et al. 1998; Hariri and Holmes 2006; Wellman et al. 2007). A complete 5-HTT knockout does not occur in humans, but a mouse model using this mutation is widely used to study the effects of serotonin deficiency. The 5-HTT protein of this genetically engineered mouse line is dysfunctional and truncated because of mRNA splicing and can only be found in the endoplasmic reticulum of serotonergic neuron soma and dendrites (Ravary et al. 2001). Even though serotonin synthesis in 5-HTT KO mice is increased, the concentration within brain tissue is significantly lowered. Reuptake from the synaptic cleft and serotonin transporter binding sites are missing, due to dysfunctional structure and subcellular localization of the truncated protein (Bengel et al. 1998; Kim et al. 2005). Mice heterozygous for the *5-Htt* gene show a phenotype more realistic and comparable to the situation of two short *5-HTTLPR* variants in humans. 5-HTT HET mice without additional stressors often show a similar behavioral performance compared to WT animals (Adamec et al. 2006; Kästner et al. 2015). From early on, 5-HT concentration in KO animals is altered drastically, leading to a highly increased concentration in the extracellular space of brain regions like the striatum, prefrontal cortex and the substantia nigra (Fabre et al. 2000; Shen et al. 2004). However, overall 5-HT levels in brain tissues are significantly decreased (Bengel et al. 1998; Kubik, Miyashita, and Guzowski 2007). 5-HT receptor alterations at plasticity level become apparent in the amygdala of KO mice, where spinogenesis is higher (Chiu et al. 1988; Schmitt et al. 2003; Schmitt et al. 2007; Nietzer et al. 2011). When investigated in paradigms established to investigate anxiety-like behavior, 5-HTT KO mice were found to be generally more anxious, whereas HET mice were not (Holmes et al. 2003; Heiming et al. 2009; Kloke et al. 2013). In addition to generally increased anxiety-like and depressive behavior (Murphy and Lesch 2008), symptoms are aggravated if mice are exposed to stress (Wellman et al. 2007; Heiming et al. 2009; Jansen et al. 2010). 5-HTT KO mice showed reduced activity levels (Holmes, Murphy, and Crawley 2002; Lewejohann et al. 2010) If exposed to paradigms including fear conditioning, learning and/or extinction, 5-HTT KO mice exhibit elevated levels of fear recall (Wellman et al. 2007; Narayanan et al. 2011).

1.3.2.1 5-HTT KO mice and prenatal stress

Different forms of stress, especially prenatal and other forms of early life stress, can also impact the susceptibility and development of depression and anxiety-related disorders (Van den Bergh et al. 2008; Van Lieshout and Boylan 2010; Weinstock 1997), as well as other psychiatric disorders (van Os and Selten 1998; Linnet et al. 2003). Individuals who experienced neglect or abuse during their early childhood, show higher rates of psychiatric diseases (de Wilde et al. 1992; Heim and Nemeroff 2002). It is known that as early as during fetal development, outside factors can influence the development of future diseases. First attempts in this direction were studies that investigated the influence of famine on the development of individuals born within or shortly after this phase. Looking at the offspring of women who were pregnant during the Dutch famine of 1944/45, it could be shown that exposition to malnourishment in early stages of gestation negatively affected the metabolism (Roseboom, de Rooij, and Painter 2006). These adaptations might be an attempt to enhance chances of survival later in life, which led to the predictive adaptive response hypothesis (PAR) (Gluckman and Hanson 2004). Malnutrition and stress during gestation permanently affects cardiac metabolism and neuroendocrine systems (Seckl and Meaney 2006; Meaney, Szyf, and Seckl 2007). An excess of stress-related hormones like glucocorticoids can lead to a reduction in birth weight and influence the developing HPA-axis (Khashan et al. 2008). However, investigating the direct influence of prenatal stress on the development of diseases later in life poses some difficulties. The time span between the perception of stressful events in the womb until birth and onset of diseases is often years, which makes it impossible to rule out other influencing factors. It is also very hard to assess how stressful experiences of the mother are perceived by and "transferred" onto the unborn child. After birth, a plethora of other influencing factors and experiences come into play, which is why considering the environment is very important. Humans are born with a unique set of genetic traits, which is also affecting the risk and course of developing future diseases.

Prenatal stress paradigms for rodents include a variety of adverse stimuli to induce stress, ranging from restraint (Ward et al. 2013; Metz, Jadavji, and Smith 2005), unpleasant sensory inputs like odors (St-Cyr et al. 2017), noise (Jafari, Mehla, et al. 2017), visual cues (Jafari, Faraji, et al. 2017) and shaking (Katz 1982) to forced exposure to water and swimming (Metz, Jadavji, and Smith 2005; Ward et al. 2013). The effects of the stress concern numerous areas of development regarding brain structure and behavior, as well as bodily functions (Beydoun and Saftlas 2008; Weinstock 2008, 2017). Alterations in birth weight as well as increased mortality of pups have been reported (Cabrera et al. 1999; Mueller and Bale 2006). Changes regarding brain development result in learning and memory deficits (Hayashi et al. 1998; Gué et al. 2004), dysregulation of the HPA-axis (van den Hove et al. 2010), a disrupted circadian clock (Yun et al. 2020), as well as a range of psychiatric disorders (Fride et al. 1986; Milberger et al. 1997). Prenatally stressed mice show elevated levels of anxiety-like behavior (Miyagawa et al. 2011; Akatsu et al. 2015). However, there are also studies that did not see changes in anxiety-related behavior after prenatal stress treatment (Nishio, Tokumo, and Hirai 2006). Moreover, one has to keep in

mind that the transferability from animal to human is difficult due to the very different perception of stress and the source of stressors. One also has to be careful to not endanger the pregnancy and general development of the pups.

5-HTT KO and HET mice are more susceptible to the negative effects of stress than WT animals (Wellman et al. 2007; Carroll et al. 2007; Jansen et al. 2010), which makes them a good candidate to study the effects of prenatal stress on individuals vulnerable to stress exposure. In her PhD-thesis, (Schraut 2015) introduced a model of prenatal restraint stress combined with exposure to bright light and water, adapted from (Behan et al. 2011) (figure 4). Pregnant dams were put in a glass cylinder of 25cm height that was filled with 0.5cm water on the bottom. A bright light source was put above the cylinder. Dams were kept in the cylinder for 45min, and the procedure was performed 3 times daily between embryonic day 13 (E13) and E17. Dams and litters were then left undisturbed until weaning. Female offspring was subjected to a battery of behavioural tests, including the 3-chamber sociability test. Based on the results of this test, animals were then split into a vulnerable, unsocial group and a resilient, social group (Schraut 2015).

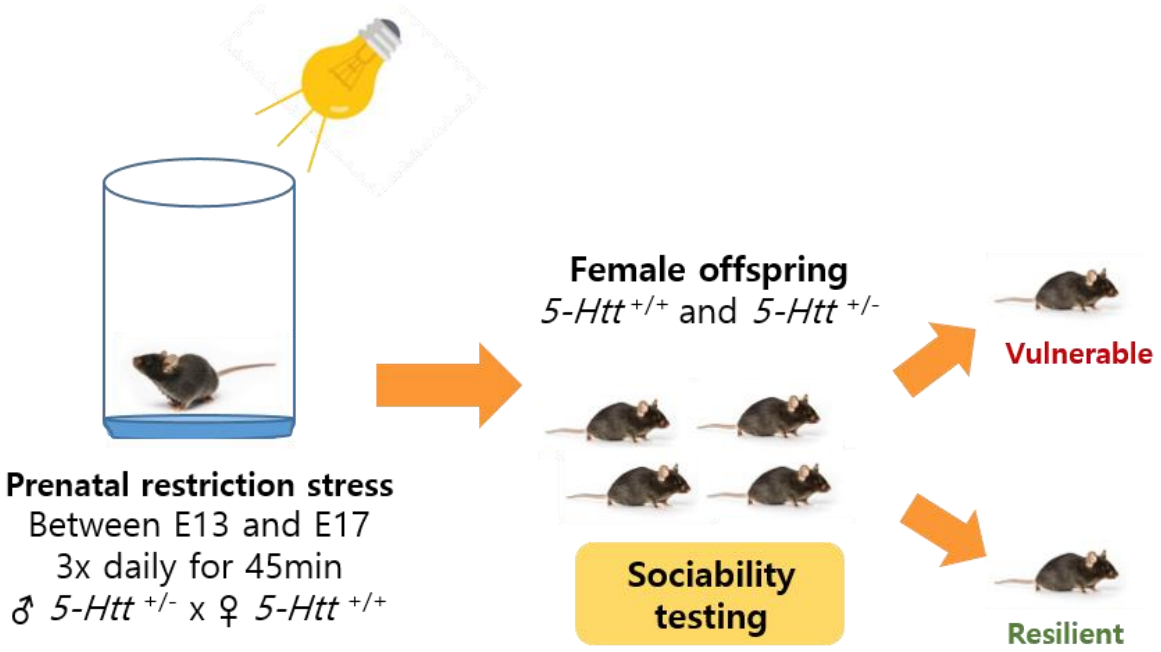

Figure 4: Experimental setup of the prenatal stress paradigm
Pregnant females were exposed to restraint stress in a glass cylinder of 25cm height, filled with 0.5cm of water and exposed to bright light. The procedure was performed three times per day for 45 minutes between E13 and E17. Female offspring was subjected to sociability testing with the 3-chamber sociability test. Based on the results of this test, animals were grouped into "vulnerable" or "resilient" offspring concerning the effects of prenatal stress on their social performance. The paradigm was used by (Schraut 2015) and is adapted from (Behan et al. 2011). Figure adapted from (Schraut 2015).

1.3.3 Vasopressin and oxytocin brain systems

Vasopressin (AVP) and oxytocin (OXT) are two peptide hormones mainly synthesized in neurons within the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus (Zimmerman et al. 1984; Gimpl and Fahrenholz 2001; Møller 2021). Magnocellular neurons of both PVN and SON project into the posterior pituitary gland, where release into the systemic circulation takes place (Yoshida et al. 2009). Parvocellular neurons seated in the PVN project to the median eminence. Special hypothalamic-hypophyseal portal vessels transport AVP into the anterior pituitary (Koshimizu et al. 2012). Known other sources of AVP are the medial amygdala and the bed nucleus of the stria terminalis (BNST) (Frank and Landgraf 2008). Additionally, there have been indications of neurons containing AVP mRNA in hippocampal regions of rats (Hallbeck, Hermanson, and Blomqvist 1999) and the presence of mRNA in the hippocampus of mice has been detected (McAdams et al. 2015). Magnocellular neurons of the PVN project OXT fibers to the nucleus accumbens (NAcc), where extracellular OXT release happens (Ross et al. 2009). Populations of OXT cell bodies are also found in the medioventral BNST (Steinman et al. 2016). OXT neurons receive input from the raphe region, which indicates possible interaction points with the serotonergic system (Althammer, Eliava, and Grinevich 2021). Structurally, both peptides are very similar, with a nonapeptide construct that only differs in the position of two amino acids. The oxytocin receptor (OXTR) is expressed in a variety of different cells and tissues and various brain areas (Yoshimura et al. 1996; Vaccari, Lolait, and Ostrowski 1998). Vasopressin has three different receptors, two of them are significantly expressed in the brain: vasopressin receptor 1a (AVPR1A) and vasopressin receptor 1b (AVPR1B). Vasopressin receptor 2 (AVPR2) can almost exclusively be found in the periphery, with the main location of expression being the renal collecting ducts (Frank and Landgraf 2008). It is expressed in the renal tubule, where it regulates water-reuptake, and in vascular endothelial cells, where it regulates vasoconstriction. AVPR1A in the periphery is expressed in vascular walls of varying types, where it regulates vasoconstriction through AVP (Share 1988). AVPR1B is very scarcely distributed in the brain, with the strongest expression found in the hippocampal region (Young et al. 2006) and some expression in the hypothalamus (Vaccari, Lolait, and Ostrowski 1998), from where it stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary (Sugimoto et al. 1994; Birnbaumer 2000). Peripheral release of oxytocin stimulates labor and milk ejection (Yoshida et al. 2009). In the brain, AVP and OXT are stored in vesicles before being centrally released in order to regulate neuronal processes (Neumann and Landgraf 2012). Their release can happen either through axon terminals of synapses or from dendritic (Ludwig 1998; Bergquist and Ludwig 2008), somatic or other axonal regions (Landgraf and Neumann 2004). AVP and OXT can travel along axonal projections to other brain areas (figure 5), where they modulate other neurons or function as neurotransmitters (Meyer-Lindenberg et al. 2011). In the amygdala, AVP and OXT modulate different neuronal populations and in consequence, modulate the fear response (Huber, Veinante, and Stoop 2005).

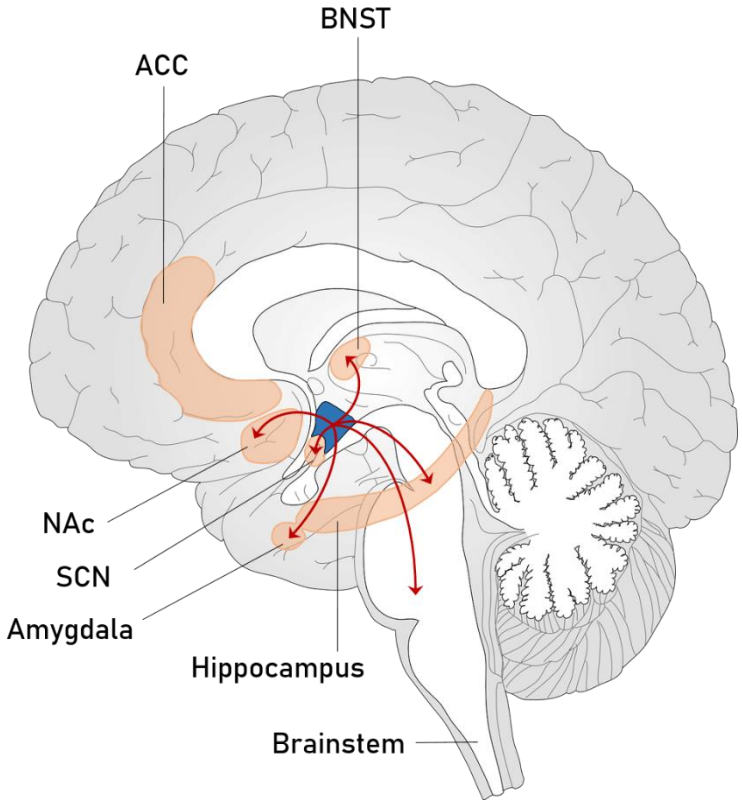

Figure 5: Axonal projections from parvocellular neurons in the PVN to other brain areas. AVP and OXT travel along axonal projections from the hypothalamus into different brain regions. ACC, anterior cingulate cortex; BNST, bed nucleus of the stria terminalis; NAcc, nucleus accumbens; SCN, suprachiasmatic nucleus. Adapted from (Meyer-Lindenberg et al. 2011).

Both AVP and OXT also have varying regulatory functions within a diverse range of social behaviors. OXT influences sexual-, and maternal behavior and harbors anxiolytic functions (Richard, Moos, and Freund-Mercier 1991; Neumann, Torner, and Wigger 2000), whereas AVP regulates aggression, social recognition, pair bonding and anxiety-like behavior, as well as anxiogenic effects (Goodson and Thompson 2010; Pagani et al. 2015). Investigation of plasma levels revealed that high concentrations of OXT are associated with a range of positive consequences, for instance low hormonal response to psychological stress factors (Taylor et al. 2006) or the result of physical contact with a partner (Grewen et al. 2005). In depressed patients, high plasma levels of OXT were associated with lower anxiety levels (Scantamburlo et al. 2007). Injection of OXT into the PVN and amygdala regions lowered anxiety levels as well as amygdala activity in male animals (Blume et al. 2008; Peters et al. 2014), whereas intracerebroventricular injections into prelimbic regions also lowered anxiety levels in female animals (Sabihi et al. 2014; de Jong, Beiderbeck, and Neumann 2014). In the periphery, low oxytocin levels were found in patients suffering from psychiatric disorders like autism (Green et al. 2001), borderline personality disorder (Bertsch et al. 2013), depression (Cyranowski et al. 2008) and schizophrenia (Goldman et al. 2008). AVP and OXT have also been related to the etiology of different psychiatric disorders, like schizophrenia (Jobst et al. 2014), depression (Yuen et al. 2014) and autism (Yang et al. 2010; Xu et al. 2013; LoParo and

Waldman 2015). However, the possible connection between peripheral and CNS neuropeptide function of AVP and OXT is not yet clear (Meyer-Lindenberg et al. 2011).
Social cognition, the ability to recognize, process and act on information about conspecifics, or social stimuli, is also regulated and modulated through AVP and OXT (Lu et al. 2019). The most prominent animal model for the role of AVP and OXT and their involvement in pair bonding is the prairie vole, a rodent that forms monogamous relationships (Carter and Getz 1993). Temporary administration of OXT leads to a significant preference for familiar conspecifics or objects both in humans and rodents, with stronger effects being observed in females (Lu et al. 2019). Partner preference in prairie voles is regulated through AVP and AVPR1A activity, especially in male individuals (Lim and Young 2004; Winslow et al. 1993). AVP seems to play a strong role in male social behavior in general, especially pair bonding behavior (Young and Wang 2004). While both AVP and OXT are regulating behavior regarding partner preference, especially preference towards familiar partners, OXT seems to be more important (Lu et al. 2019). Interestingly, the preference is not only limited to possible sexual partners, but also involved in same-sex partner preference (Beery and Zucker 2010; Bales et al. 2013).
Human studies investigating the influence of intranasal OXT administration showed facilitated encoding and retrieval of recognition memory (Weigand et al. 2013) and the recall of social affiliation memories (Cardoso et al. 2014) in male individuals, as well as an increase of communicative behavior during couple conflicts in both sexes (Ditzen et al. 2009). Interestingly, the suppressing effect of OXT on stress hormone levels like cortisol after intranasal application was reduced in adult men who experienced parental separation stress early in their life (Meinlschmidt and Heim 2007). This might indicate that the OXT central system and its functions are established early in life (Meinlschmidt and Heim 2007). AVP administration elevated general levels of cognitive function in both young (Beckwith, Couk, and Till 1983) and elderly (Jennings, Nebes, and Reynolds 1986) people. However, the possible influence of OXT on memory formation and recall remains mostly unknown. Animal studies were able to show that AVP is also involved in the regulation of memory (Barsegyan et al. 2015; Veenema, Bredewold, and De Vries 2012). The regulation of social behaviors through AVP and OXT shows a lot of similarities to the regulation of the hypothalamic-pituitary-adrenal (HPA) axis activity (Baribeau and Anagnostou 2015). First studies comparing the effects of AVP and OXT point to opposite functions. While AVP seems to improve cognitive performance levels, OXT might impair processes involved in learning and memory (Fehm-Wolfsdorf et al. 1984). It is assumed, that the concentrations of vasopressin and oxytocin brain systems are somewhat in balance while regulating social behavior and high concentrations of either one lead to extreme behavioral outcomes on both ends of the anxiety spectrum, with high vasopressin concentrations leading to hyper-anxious states and high oxytocin states leading to hypo-anxious states (figure 6), (Neumann and Landgraf 2012).

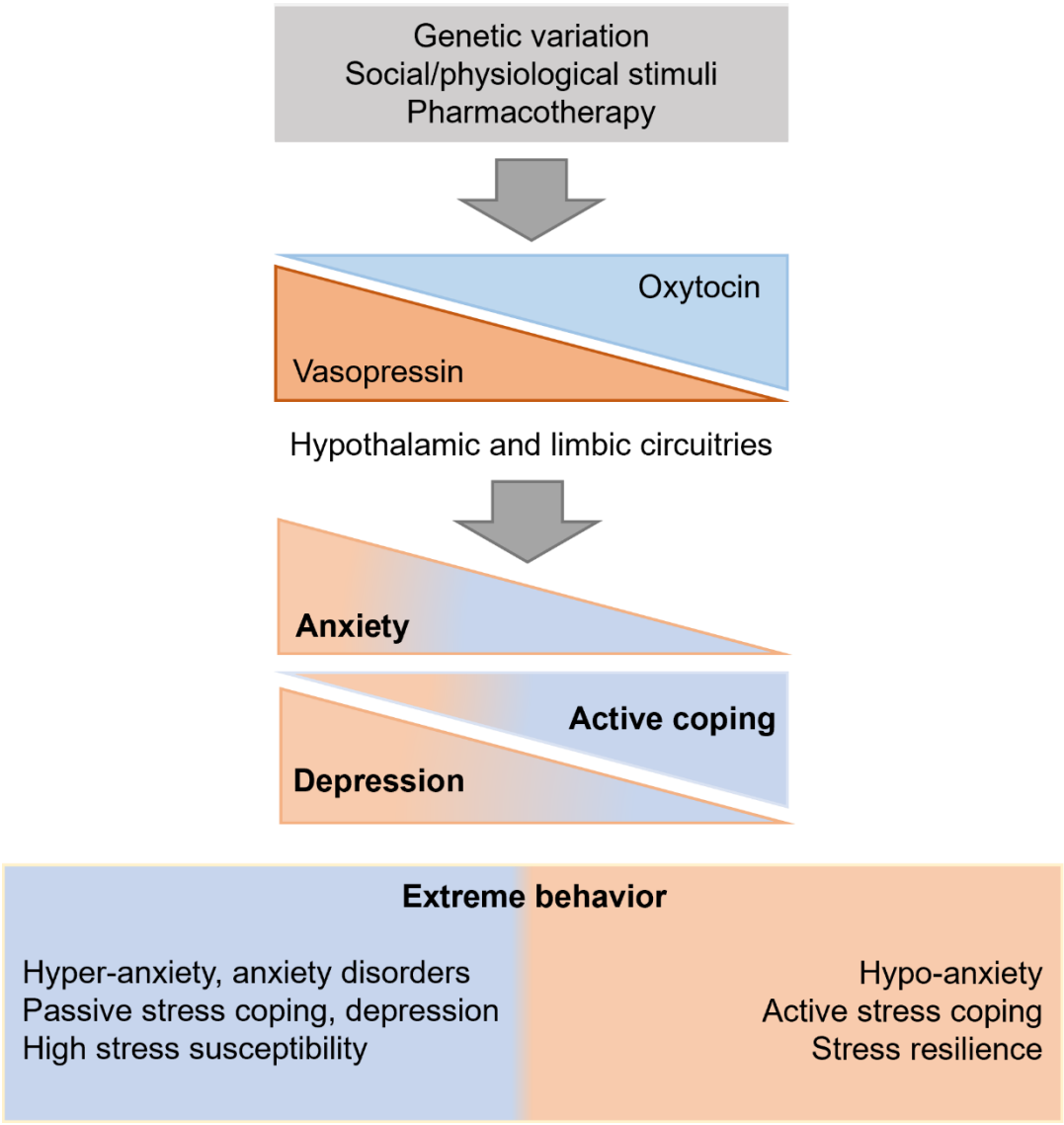

Figure 6: Brain balance model between vasopressin and oxytocin.
Vasopressin and oxytocin release and concentration are stimulated through different epi-genetic factors and the application of pharmacotherapy. The supposedly opposing effects of their release influence social behavior in response to stress. If the concentration is off balance, it can lead to extreme behavioral effects on both sides of the spectrum. High vasopressin concentrations are associated with states of hyper-anxiety and anxiety-related disorders and depression, whereas high oxytocin concentrations lead to states of hypo-anxiety and extreme stress resilience. Adapted from (Neumann and Landgraf 2012).

1.3.4 Neuropeptide Y in the brain

Neuropeptide Y (NPY) is involved in the regulation of varying physiological functions like the circadian rhythm (Morin 2013) and energy homeostasis (White 1993). It is also responsible for stress modulation, where it displays anxiolytic and fear reducing abilities (Reichmann and Holzer 2016; Heilig 2004). Synthesis of NPY happens in different brain regions, but has mainly been identified in the septohippocampal nucleus, locus coeruleus, and the arcuate nucleus (Kask, Vasar, et al. 2001). NPY can also be found in PVN, hippocampus and amygdala (Broberger et al. 1999; Kask, Vasar, et al. 2001; Holzer,

Reichmann, and Farzi 2012). In vertebrates, seven different NPY receptors (NPYRs) have been identified so far. Five of them are functional in mammals (NPYR1, 2, 4 and 5) (Reichmann and Holzer 2016). NPY shows the highest affinity to its receptors NPYR1, 2 and 5 (Alexander et al. 2013). While NPYR4 expression is very scarcely distributed throughout the brain (Kask et al. 2002), NPYR5 expression is limited to limbic areas (Morales-Medina, Dumont, and Quirion 2010). NPYR1 and 2 are abundantly expressed in the brain and can be found in the lateral septum, lateral hypothalamus, amygdala and hippocampus among other regions (Kopp et al. 2002; Kask et al. 2002; Eva et al. 2006). NPYR2 is strongly expressed in the septum region (Harro 2006). It is believed that NPY is involved in terminating the stress response through acting upon the HPA-axis (Heilig et al. 1994). Injected intracerebroventricularly, NPY harbors anxiolytic effects (Heilig et al. 1989). This can also be observed after injection into hippocampus (Śmiałowska et al. 2007) and amygdala (Sajdyk et al. 2008), which hints towards an involvement of both regions in the process. The anxiolytic effects of NPY are supposedly mediated through NPYR1 (Primeaux et al. 2005; Sørensen et al. 2004), whereas the NPYR2 seems to harbor mostly anxiogenic effects (Nakajima et al. 1998; Kallupi et al. 2014). As part of the response to stress, an increase in anxiety is triggered through corticotropin releasing hormone (CRH). This effect can be counteracted through NPY (Śmiałowska et al. 2007; Sajdyk et al. 2008). Administration of NPY can reduce induced fear through simultaneous activation of the postsynaptic NPYR1 and the presynaptic NPYR2 (Kornhuber and Zoicas 2019). This is mediated through NPYR1 activation in the central amygdala and NPYR2 in the dorsolateral septum (Kornhuber and Zoicas 2021b).

1.3.5 Relevant brain regions

Due to limited resources, we had to limit our experiments to selected brain regions that could be investigated in our studies. The hypothalamus, and more specifically the PVN, has the most importance for the AVP and OXT systems, due to it being the source of their production.

In the context of fear and anxiety disorders, the amygdala is a very important region to investigate. As part of the limbic system, this almond-shaped group of nuclei is involved in emotional regulation and learning (Anderson and Phelps 2001; Feinstein et al. 2011), as well as perception, learning and expression of fear (Fanselow and LeDoux 1999). Furthermore, the amygdala is targeted by magnocellular AVP- and OXT-containing neurons from the PVN (Møller 2021).

The hippocampus is also a part of the limbic system and important for learning processes, especially the consolidation from short- to long-term memory and its storage, as well as the formation of declarative memory (Squire 1992). It also processes context information, for example the environment connected to a fearful situation (Kim, Rison, and Fanselow 1993). The functional capacities of the human hippocampus differ alongside its anterior-posterior/dorsal-ventral axes (Moser and Moser 1998). Due to the anatomical and functional differences between the human and the murine brain, the denomination for the axes will be different in order to distinguish between the species. The hippocampal axes

of the human brain will be referred to as anterior/posterior. The hippocampal axes of the murine brain will be referred to as dorsal/ventral. The posterior hippocampus of humans, corresponding to the dorsal hippocampus of mice, is mostly responsible for cognitive functions like spatial learning (for review see (Zeidman and Maguire 2016)). The ventral parts of the hippocampus in mice are believed to mediate stress responses (Fanselow and Dong 2010; Bannerman et al. 2004).
The raphe nuclei, as the source of serotonin production, are important in every context of anxiety disorders, because of the key regulating functions of serotonin regarding mood and anxiety (Lucki 1998). Through the dorsomedial hypothalamic nuclei, the midbrain raphe nuclei provide reciprocal feedback to the hypothalamic suprachiasmatic nuclei, a mechanism involved in the regulation of circadian rhythms (Deurveilher and Semba 2008).
The septum region consists of a number of nuclei, which can be divided into four groups, based on their anatomical location: lateral, medial, posterior and ventral (Risold 2004). The lateral group consists of the lateral septal, septohippocampal and the septofimbrial nuclei (Risold 2004). The largest of the nuclei is the septal nucleus, which can be subdivided into dorsal, intermediate and ventral parts, based on cell size and density differences along its dorsoventral axis (Swanson and Cowan 1979; Paxinos and Watson 2009). The septum is involved in the regulation of emotional-, social- and fear-related behavior (Sheehan, Chambers, and Russell 2004). Intracerebroventricular administration of OXT reduces the effects of induced social fear, mediated through the OXTR in the dorsolateral septum (DLS) (Zoicas, Slattery, and Neumann 2014; Menon et al. 2018). Additionally, social fear leads to an increase of OXTR binding in the dorsolateral septum (Zoicas, Slattery, and Neumann 2014).

1.4 Aim of the thesis

The general aim of this thesis was to evaluate possible relationships between the serotonergic and the AVP- and OXT- brain systems and their possible interactions within the context of different fear- and anxiety related disorders. We covered the analysis with two different main projects. The first main project focused on SFC, an animal model of SAD, researching its effects on brain areas involved in social- and fearful behaviors. With a gene expression study covering IEGs Arc, cFos and Fosl2 as markers for neuronal activation and several genes related to AVP-, OXT-, NPY-, and the serotonin system, we were able to highlight the effects of SFC on mRNA expression. In a second approach in this study, we evaluated cFOS protein levels, in order to determine the changes through SFC on protein level.
The second main project involved the brains of 5-HTT deficient mice that had been subjected to a prenatal stress paradigm, as well as naïve mice of this mouse line. The original study performed by Karla Schraut utilizing the prenatal stress paradigm had already found alterations in social behavior of prenatally stressed mice and subsequently grouped the animals into social and unsocial groups, based on their performance in sociability testing. In an own initial study with female offspring, we investigated expression of genes related to the AVP-, OXT- and serotonin brain systems to search for

alterations caused through prenatal stress exposure in combination with 5-HTT deficiency. Following the preliminary results of this study, we then investigated the brains of male mice that were exposed to the prenatal stress paradigm and naïve male and female mice of different *5-Htt* genotypes, to further corroborate the initial results. In addition to that, in a smaller side project we investigated the number and density of AVP- and OXT-positive cells in mice of both sexes and all three different *5-Htt* genotypes, to look for possible alterations on protein level.

2 Materials and Methods

2.1 SFC-project

2.1.1 Animals and ethics

Behavioral experiments and animal handling was performed in collaboration with Iulia Zoicas from the University of Erlangen. Forty 8-week-old male C57BL/6J mice (purchased from Charles River, Sulzfeld, Germany) were individually housed for three days before the experiment started and remained single-housed throughout the experiment. Mice were kept under standard laboratory conditions (12:12 light/dark cycle, lights on at 07:00 h, 22°C, 60% humidity, with food and water *ad libitum*). Experiments were performed during the light phase between 09:00 and 14:00 in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of Unterfranken (approval code 55.2 DMS-2532-2-314, approval date 13.12.16) and the guidelines of animal care and use by the European Community.

Social Fear Conditioning and fear assessment

To induce social fear, mice were conditioned during SFC using a computerized fear conditioning system (TSE System GmbH, Bad Homburg, Germany), as developed by (Toth, Neumann, and Slattery 2012b). Mice were placed in the conditioning chamber (45 x 22 x 40 cm), and after a 30-sec habituation period, an empty wire mesh cage (7 x 7 x 6 cm) was placed as a non-social stimulus near one of the short walls. After 3 min, the non-social stimulus was replaced by an identical cage containing an unfamiliar age- and sex-matched mouse. Unconditioned mice (SFC-) could investigate this social stimulus for 3 min, whereas conditioned mice (SFC+) were given a 1-sec mild electric foot shock (0.7 mA) each time they made direct contact with the social stimulus. Mice received two foot-shocks with a variable inter-shock interval, depending on when direct social contact was made. The number of foot shocks was assessed as a measure of distress and of social fear learning. Mice were returned to their home cage when no further social contact was made for 2 min (average duration of SFC approximately 10 min). The time that the mice spent investigating the non-social stimulus as a pre-conditioning measure of non-social anxiety was analyzed. The SFC-induced social fear was assessed one day after SFC. Mice were exposed in their home cage to a small wire mesh cage containing an unfamiliar mouse for 5 min, to assess social investigation as a parameter of social fear. The stimulus was placed near a short wall of the home cage. The test was recorded and analyzed using JWatcher. Social investigation was defined as direct sniffing of the cage and/or of the social stimulus inside of the cage. A schematic overview of the experimental procedures is shown in figure 7 below.

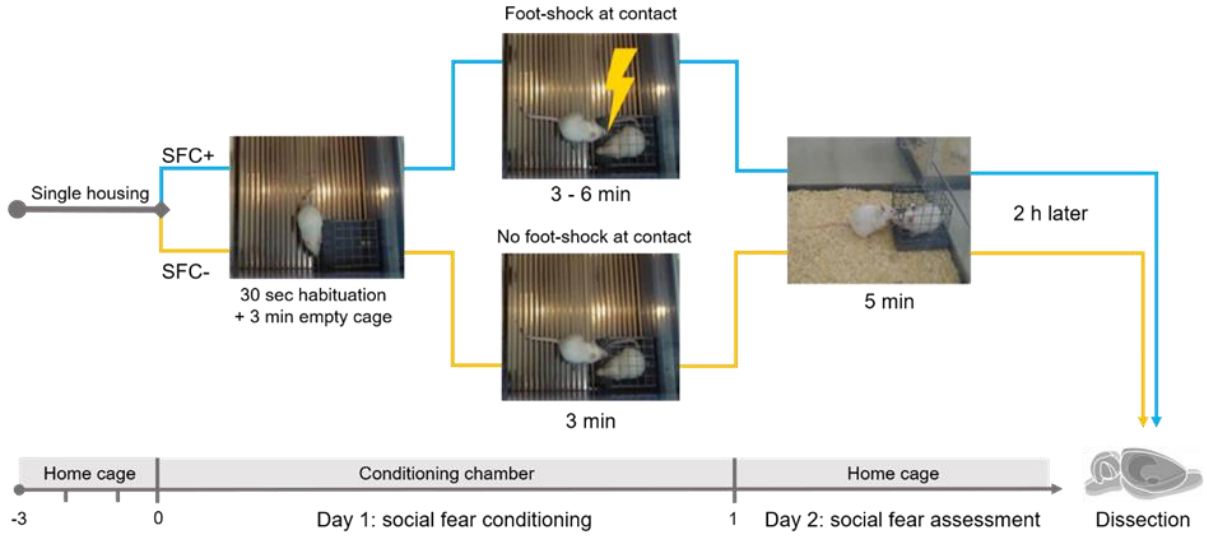

Figure 7: Schematic overview of Social Fear Conditioning experimental procedures. Animals were single housed for 3 days before being exposed to the SFC setup. Animals were divided into two different groups, both groups first passing through a habituation phase. After initial habituation, SFC+ groups received a foot-shock while trying to investigate an unknown conspecific, while SFC- groups did not receive a shock. After approximately 5 minutes (SFC+ group) or 3 minutes (SFC- group), animals were put back into their home cage until the next day. On the second day of the SFC paradigm, all experimental animals were exposed to an unfamiliar conspecific in their home cage for 5 minutes. 2 hours after the exposure, animals were sacrificed, and brains were dissected. This figure was created with J. Bankmann and is part of his Master-Thesis in an adapted version (Bankmann 2020).

2.1.2 Tissue preparation

Two hours after exposure to the social stimulus on day 2 of the SFC paradigm, mice were decapitated under a very short anesthesia caused by CO2 intake. Blood for corticosterone measurements was taken directly from the heart or, in case this was not possible, trunk blood was collected from the site of decapitation. Samples were collected in heparinized blood collection tubes (Sarstedt, Nümbrecht, Germany), put on ice and centrifuged for 5 min at 3000g and 4°C. The separated plasma was put in new collection tubes and stored at -80°C until evaluation. Determination of corticosterone levels was performed via radioimmunoassay according to the protocol previously described in (Van den Hove et al. 2006). Whole dissected brains used for gene expression analysis were directly snap-frozen in pre-cooled isopentane. Brains being used for immunohistochemistry were fixed by immersion in 4% paraformaldehyde (PFA) (dissolved in 0.1 M PBS, pH 7.5) for 48 h and then cryoprotected using 10% and 20% sucrose (dissolved in 0.1 M PBS, pH 7.5) for 24 h, consecutively. After cryoprotection, brains were also snap-frozen in pre-cooled isopentane and stored at -80°C.

2.1.3 Gene expression

Primers

Primer sequences were chosen using the NCBI BLAST tool. Primers were purchased from Sigma-Aldrich (St. Louis, USA).

Table 1: List of primers used for the SFC qPCR-study

Reference Gene	Full name	Product [bp]	RefSeq.	Primer-Sequence
Actb	Beta-actin	84	NM_007393.5	F: ATGTGGATCAGCAAGCAGGA R: AGCTCAGTAACAGTCCGCCTA
B2M	Beta-2 microglobulin	126	NM_009735.3	F: ACCGTCTACTGGGATCGAGA R: TGCTATTTCTTTCTGCGTGCAT
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase	135	NM_008084	F: GTGATGGGTGTGAACCACGA R: GGTCATGAGCCCTTCCACAA
Gdi2	Rab GDP dissociation inhibitor beta	126	NM_008112	F: GTCAGAATTGGTTGGTTCTGTTC R: AGCTCTTGGATCACACAATCG
Rplp0	60S acidic ribosomal protein P0	83	NM_007475	F: GAGGCCACACTGCTGAACAT R: ATGCTGCCGTTGTCAAACAC
Gene of interest				
Arc	Activity-regulated cytoskeleton-associated protein	82	NM_018790	F: GGGTGAGCTGAAGCCACAAA R: ACTGGTATGAATCACTGGGGG
Avp	Vasopressin	125	NM_009732.2	F: CAAGAGGCGGCAAGAGGG R: CACGAAGCAGCCCAGCTC
Avpr1a	Vasopressin receptor 1A	114	NM_016847.2	F: CCTACATCCTCTGCTGGACAC R: CCAGTAACGCCGTGATCGT
cFos	Fos proto-oncogene	149	NM_010234.3	F: GGCAGAAGGGGCAAAGTAGAG R: TCAAGTTGATCTGTCTCCGCTTG
Fosl2	Fos-related antigen 2	107	NM_008037.4	F: GGTTTCTACGGGGAAGAGCC R: TCCAGGACATTGGGGTAGGT
Htr1a	Serotonin 1A receptor	144	NM_008308.4	F: GATCTCGCTCACTTGGCTCA R: AAAGCGCCGAAAGTGGAGTA
Htr2a	Serotonin 2A receptor	112	NM_172812.3	F: CCATAGCCGCTTCAACTCCA R: CGAATCATCCTGTAGCCCGA
Htr2c	Serotonin 2C receptor	73	NM_008312	F: GCAATAATGGTGAACCTGGGC R: ACTGCCAAACCAATAGGCCA
Npy	Neuropeptide Y	106	NM_023456.3	F: CAGATACTACTCCGCTCTGCGACACTACAT R: TTCCTTCATTAAGAGGTCTGAAATCAGTGTCT
Npyr1	Neuropeptide Y receptor Y1	105	NM_010934	F: ATTTCGGCCCACTCTGCTTT R: ACCTGTACTTACTGTCCCGGA
Npyr2	Neuropeptide Y receptor Y2	77	NM_008731	F: CATCTGAGAAGGAACGCGCA R: CTACCGGGCCCATCTTCAGA
Oxt	Oxytocin	141	NM_011025	F: GAGGAGAACTACCTGCCTTCG R: CGAGAAGGCAGACTCAGGGT
Oxtr	Oxytocin receptor	123	NM_001081147	F: ACGTCAATGCGCCCAAAGAA R: GCACGAGTTCGTGGAAGAGAT
Tph2	Tryptophan hydroxylase 2	184	NM_173391	F: TGGGGATTTGATGCCTAGAACC R: TGGGTTCTTTAGAGCATTTTTGTGT

Buffers, solutions and material

Table 2: Buffers, solutions, material and equipment used for the gene expression study

Buffer	**Composition**
Phospate buffered saline (PBS) 1x	10x PBS (Lonza) 1:10 in ddH_2O
Blocking Buffer I	5% Normal Goat Serum in PBS
Sodium Citrate Buffer pH 6	1 mM Tri-Sodium-Citrate in ddH_2O 0.1 M HCl for titration
Solution	**Company/Composition**
Chloroform > 99% p.a.	Carl Roth
Cresyl Violet Acetate	Merk 0.1% Cresyl Violet Acetat (321.34 g/mol) for vaginal lavage 0.25% Cresyl Violet Acetat (321.34 g/mol) for laser capture microdissection in ddH_2O
CryoGel	Leica
Ethanol absolute	AppliChem
Hydrogen Peroxide 30%	Merk
Qiazol	Qiagen
RNAse AWAY®	ThermoFisher
RNAprotect tissue reagent	Qiagen
SYBR Select Mastermix	Applied Biosystems
Vitro-Clud	R. Langenbrinck
Material	**Company**
PEN membrane slides 2µm	Leica
Silica Gel Orange 2-5mm	Carl Roth
Equipment	**Company**
Bioanalyzer 2100	Agilent
CFX 1000 Touch 384 cycler	BioRad
Cryostat CM 1950	Leica
IKA MS3 digital mixer	IKA
LMD 6000	Leica
TProfessional Basic Thermocycler	Biometra

Kits

Table 3: List of ready for use kits utilized for the SFC gene expression study

Kit	**Company**
miRNeasy Micro Kit 50	Qiagen
RNA 6000 Nano Kit	Agilent

RNase free DNase set	Qiagen
5x iScript cDNA synthesis kit	BioRad
2x SsoAdvanced PreAmp Supermix	BioRad

Table 4: List of software used for the SFC gene expression study

Software	**Company**
2100 Bioanalyzer Expert	Agilent
CFX Maestro 2.0	BioRad
LinRegPCR	Ruijter et al. 2009
LMD software 7.6	Leica
PRISM 6.0	GraphPad
qbase+	Biogazelle
SPSS 26	IBM

Laser capture microdissection

Frozen native brain tissue was sectioned to 20 µm in a Leica CM1950 freezing microtome at 20 °C and mounted on polyethylene naphthalate (PEN) membrane slides 2.0 µm (Leica, Wetzlar, Germany). Mounted tissue sections were fixed in 75% EtOH (diluted in DEPC-treated water) for 2 min, stained with 0.25% cresyl violet and then processed through an ascending ethanol series (75 %, 90 %, 100 % ethanol for 20 sec each) for decoloring. After an additional incubation for 1 min in 100 % ethanol the slides were air dried in 50 ml tubes with Silica Gel Orange, (Roth) and tissue was dissected using a Leica LMD6 laser microdissection system (Fig. 1). Brain regions of interest were the pyramidal cell layer (pcl) of cornu ammonis area (CA)1 and 3, granule cell layer (gcl) of the dentate gyrus (DG), Septum, the basolateral amygdala (BLA), the paraventricular nucleus of the hypothalamus (PVN), and the dorsal raphe (DR). RNA was extracted from collected tissue using the miRNeasy Micro Kit (Qiagen, Hilden, Germany), following manufacturers protocol. RNA was stored at -80°C until further use. An example of the LMD workflow is shown in figure 8 below.

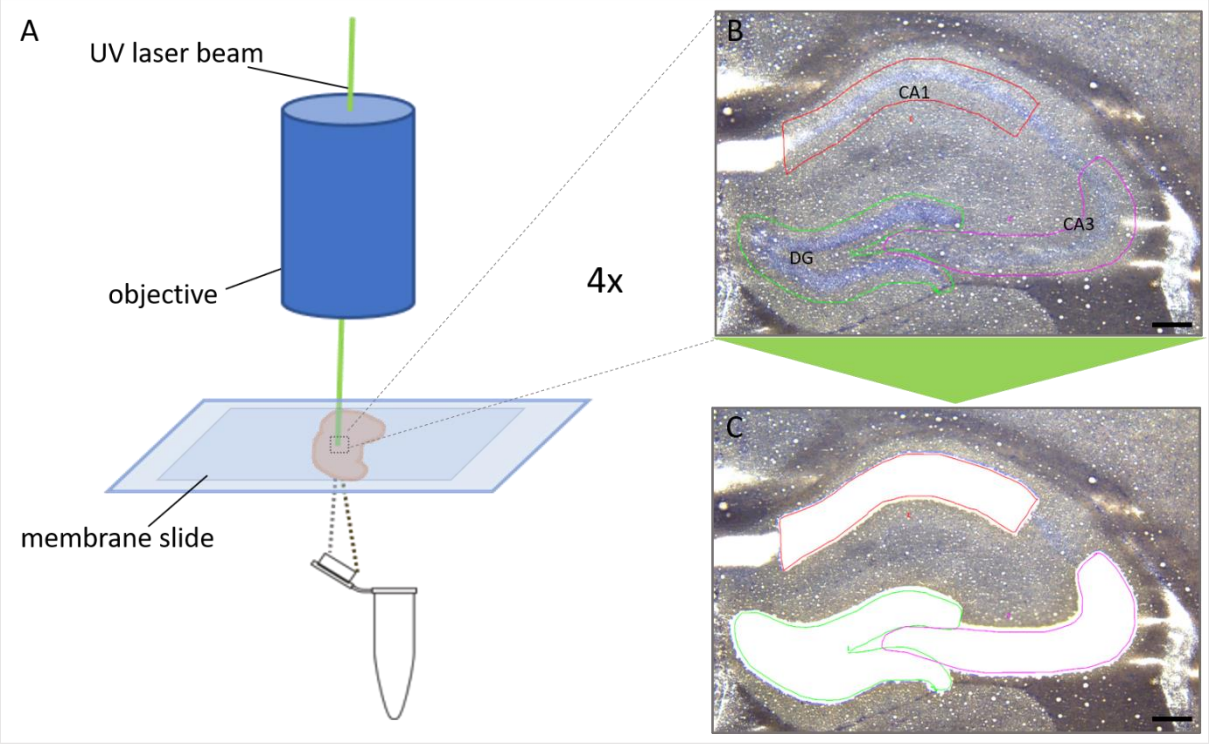

Figure 8: Workflow of the LMD procedure.
A: Schematic overview. UV laser beam is sent through a 4x objective of the microscope and cuts the PEN-membrane and tissue at the desired location. Tissue is collected in the tip of a collection tube beneath. B: Cresyl-violet staining before LMD with outlined regions of interest: pyramidal cell layer of cornu ammonis area 1 (CA1) and CA3 and granule cell layer of dentate gyrus (DG). C: the remaining tissue after successful LMD. Scale bars in B and C represent 200 µm. This figure was created in cooperation with J. Bankmann and is part of his Master-Thesis (Bankmann 2020).

RNA quantification

To precisely detect the small amount of RNA retrieved from the extraction procedure and measure its integrity, the Agilent 2100 Bioanalyzer system was used. Aliquots of 1.2µl RNA were used with the Agilent RNA 6000 Nano Kit, following manufacturers' protocol.

cDNA synthesis

In order to synthesize the cDNA needed for real- time polymerase chain reaction (qPCR) procedures from RNA, iScript cDNA synthesis Kit was used. 50ng of RNA were applied to the procedure, following manufacturers' protocol. RNA was mixed with 4 µl 5x iScript reaction mix and 1 µl iScript Reverse Transkriptase (RT) and a variable amount of RNAse free water, filling up the volume to 20 µl in total. PCR protocol was performed according to the provided protocol, shown in table 5 below.

Table 5: Protocol used for cDNA synthesis

Temperature	Time	Step
25C°	5 min	Priming
46°C	20 min	Reverse transcription
95°C	1 min	RT inactivation
4°C	∞	Stop

After the reaction was finished, cDNA was diluted 1:2 in nuclease free water and stored at -20°C until further use.

Preamplification of specific target sequences

Based on the limited amount of RNA and synthesized cDNA respectively, an amplification step to enhance the amount of specific cDNA was necessary. The 2x SsoAdvanced PreAmp Supermix was used for this procedure. All desired primers for the qPCR study were put together into a primer assay which was comprised of 10 µl of each primer taken from a 100 µM stock solution (for a detailed list, see table 6), filling up the volume with nuclease free water to 2 ml and reaching a final concentration of each primer of 500 nM/µl. The primer assay was then stored at -20°C until further use. The preamplification procedure was performed according to manufacturer's protocol, mixing 5 µl of the primer assay with 25 µl 2x PreAmp Supermix and 20 µl of 1:2 cDNA, reaching a total volume of 50 µl. The PCR was performed according to the protocol in table XY below.

Table 6: cDNA preamplification protocol

Temperature	Time	Repetition
95°C	3 min	
95°C 60°C	15 sec 4 min	10x
4°C	∞	

After the preamplification was finished, cDNA was diluted with 200 µl 1x TE-buffer to a 1:5 stock solution and stored at -20°C until further use.

Real-time quantitative polymerase chain reaction

To perform the qPCR reactions, the stock solution of preamplified qPCR target sequences was further diluted using water to a 1:20 working solution. 0,5µl forward and reverse primer (5µM) per sample were mixed with 5µl SYBR™ Select Master Mix (and 4µl cDNA to a total volume of 10µl. A 384-well plate in a CFX384 Real-Time PCR detection system (Bio-Rad) was used for the reactions. The qPCR was performed according to the protocol in table 7 below.

Table 7: protocol for qPCR with 40 cycles

Temperature	Time	Repetition
95°C	2 min	
95°C 60°C	5 sec 30 sec	40x
65°C – 95°C Increments of 0.5°C	5 sec	
4°C	∞	

LinReg PCR program (2017 version, (Ruijter et al. 2009)) was used to calculate mean PCR efficiencies. Results were normalized using beta-actin (Actb), beta-2 microglobulin (B2M), Glyceraldehyde 3-phosphate dehydrogenase (Gapdh), Rab GDP dissociation inhibitor beta (Gdi2) and 60S acidic ribosomal protein P0 (Rplp0). Evaluation of expression, normalization and calculation of relative gene expression results were obtained with qBase+ software (Biogazelle, Ghent, Belgium). Genes were excluded from the analysis, if more than 15% of the samples did not meet the evaluation criteria of less than 0.5 threshold cycles difference between the analyzed triplets.

2.1.4 Immunohistochemistry and immunofluorescence

Immunohistochemistry

Serial coronal sections were cut at 30 µm using a Leica CM1950 freezing microtome and transferred to HistoBond®+ adhesion slides. Every sixth section was used for each individual series. Sections were air dried for 45 min and washed with PBS. They were treated with 0.6% H_2O_2 in PBS for 30 min. Antigen retrieval was performed using citrate buffer for 20 min at 80°C in a water bath. Sections were blocked with 5% normal goat serum (NGS, Vector Laboratories) in PBS for 1.5 h. Incubation with the primary antibody polyclonal rabbit anti-cFOS (1:750, Proteintech, Manchester #26192-1-AP) in 10% NGS was applied overnight at 4°C. On the second day, sections were incubated for 30 min with the corresponding Histofine Simple Stain MAX PO detection system (rabbit, Nichirei Biosciences, Japan). For visualization, DAB-Peroxidase substrate (diluted 1:10 in peroxidase buffer, Vector Laboratories) was applied onto the sections for 10 min. Sections were dehydrated using ascending alcohol series and covered in Vitro-Clud for preservation.

Immunofluorescence double staining

Serial coronal sections were cut at 30 µm using a Leica CM1950 freezing microtome and transferred to HistoBond®+ adhesion slides. Every sixth section was used for each individual series. Sections were air dried for 45 min and washed with TBS. Antigen

retrieval was performed using citrate buffer (10mM Sodium Citrate, pH 6.0) for 20 min at 80°C in a water bath. Sections were blocked with 10% normal horse serum (NHS) and 1% Triton X-100 in TBS for 1h. Incubation with the primary antibodies polyclonal rabbit anti-cFOS (1:500, Proteintech) and monoclonal mouse anti-vasopressin (1:100, Santa Cruz, #sc-390723) in 5% NHS was applied 48h at 4°C. On the third day, sections were washed with PBS and incubated with the corresponding secondary antibodies donkey anti-rabbit Alexa Fluor 488 (1:400, Thermo Fisher, #A-21206) and donkey anti-mouse Alexa Fluor 647 (1:400, Thermo Fisher, #A-31571) for 1.5h. Sections were again washed with TBS and covered in Fluoro-Gel for preservation.

Imaging

Light microscope images were captured using an Olympus BX51 microscope with 4x and 10x PLAN N air objectives using an Mbf Bioscience Cx9000 camera. Stereo Investigator® software was used to process the images (MBF Bioscience, Williston, USA, software version 2018). Fluorescence images were captured using an Olympus FluoView FV1000 Confocal microscope with a 10x UPlanAPO 0.40 NA objective. Cell count and area size was measured using ImageJ software (Fiji) version 1.52n (Schindelin et al. 2012).

Quantification of immunohistochemistry results

Number of cFos-immunoreactive cells within the hippocampal areas were counted by an experimenter blinded for treatment of the animals. Cells were counted manually using an Olympus BX51microscope combined with a MBF CX 9000 camera and the corresponding motorized stage, in order to move freely around the area. For the counting procedure, Stereo Investigator Software 2020.2.1 was used. Average thickness of the tissue was measured for every individual slice. Area sizes of the pyramidal cell layer of CA1 and CA2 and the granular cell layer of the DG were measured at 10x magnification. All areas were divided into an dorsal and a ventral part, the former starting from the part of visual discriminability, the latter starting after corpus callosum separation. Cell counting was performed at 20x magnification. Approximately 6 sections belonging to the dorsal portion and 5 sections belonging to the ventral portion were used for counting, to keep the reference size comparable. Cells that didn't show a clearly positive staining were excluded from the counting procedure. Mean number of cells and cell density was calculated for each region of interest.

2.1.5 Statistical analysis

All data were analyzed using SPSS Statistics 26 (IBM Corporation, New York, USA). GraphPad Prism software version 8 (GraphPad Software, La Jolla, California, USA) was used for data graphing. Boxplots were used to visualize symmetry, skew, variance, and outliers of the data sets. Additionally, the Levene's test for homogeneity of variances and a Shapiro-Wilk test of normality were performed. Statistical analysis was performed using either a two-sample t-test or a Mann-Whitney-U test. The Mann-Whitney-U test was

applied when homogeneity of the variance and/or the normal distribution was violated. Data are shown as means ± standard errors of the mean (SEM). Differences at trend level are indicated as #p<0.1, significant differences as *p<0.05, **p<0.01, ***p<0.001.

2.2 Prenatal stress project

2.2.1 Animals and ethics

Experimental procedures regarding the 5-Htt x PS paradigm and following behavioral analyses, sacrificing of the animals and brain extraction were performed by Karla Schraut and are only described here in brief. For a detailed overview, see (Schraut 2015). The number of utilized animals for each subproject is described in the statistical analysis of the results part. *5-Htt* WT dams were bred with HET male animals, which led to WT and HET genotypes in the offspring. Experimental animals were exposed to a prenatal stress paradigm, adapted from Behan et al., 2011. Between E13 and E17 dams of the PS group were placed into a glass cylinder, which was exposed to bright light and contained water on the bottom. The procedure was repeated 3 times for 45min daily. The offspring was weaned after 25 days, and female pups introduced to behavioral testing. Male offspring was weaned and left without behavioral testing until sacrifice. Female animals were subjected to elevated plus maze, Porsolt forced swimming test, 3-chamber-sociability test and sucrose preference test. For additional experiments, naïve male and female mice of all *5-Htt* genotypes (WT, HET, KO) were utilized.

2.2.2 Extraction of brain regions

Frozen brains that had been stored at -80°C were placed into a petri dish onto a cooling plate at -8°C, to slowly start a thawing process, which made it possible to cut the tissue. With a scalpel, the brain was cut into 3 to 4 mm thin coronal sections. With the help of a stereomicroscope that is equipped with a 100-watt halogen lamp and small spatulas, the desired brain regions hypothalamus, hippocampus, amygdala and raphe were individually extracted, placed into a sterile reaction tube and frozen at -80°C until further tissue processing.

2.2.3 Vaginal lavage and cytological staining

To determine the estrous cycle stage of female animals, naïve animals of all three *5-Htt* genotypes were subjected to a vaginal lavage after being sacrificed. The protocol is adapted from (McLean et al. 2012). The outside of the vaginal canal was rinsed once with 100 µl of ddH_2O to get rid of any urine residues and a cytological sample was taken with again 100 µl of ddH_2O, spreading the cells onto a glass slide and letting them dry. Dried cells were stained with 0.1% crystal violet in ddH_2O for 1 min, rinsing in ddH_2O for 1 min. The procedure was repeated once, and the surface around the cells dried with a tissue.

Slides were then left to air-dry. Dried slides were put into an ascending alcohol series, starting from 75% EtoH, 80% EtoH, 90% EtoH, 100% EtoH, Xylene for 1 minute each and mounted using Vitro-Clud® for long-term storage.

2.2.4 DNA extraction and genotyping

Buffers and solutions

Table 8: Buffers, solutions and material used for DNA extraction and genotyping

Buffer	**Composition**
Loading buffer	10 mM Tris-HCl 0.03% Bromophenol Blue 0.03% Xylene Cyanol FF 60% Glycerol 60 mM EDTA
Lysis buffer	1,5 M Tris-HCl pH 8.5 5 M NaCl 0,5 M Ethylenediaminetetraacetic acid (EDTA) pH 8.0 20% Sodium dodecyl sulfate (SDS) ddH_2O
PCR buffer	1 M Potassium chloride (KCl) 1 M Tris-HCl, pH 8,3 0,1 M Magnesium chloride ($MgCl_2$) 10 % Tween 20 Bovine serum albumin (BSA) (100x purified) (New England Biolabs) ddH_2O
Solution	**Company**
Agarose gel	3 % Agarose in 1x TAE buffer
Chloroform >99% p.a.	CarlRoth
Ethanol absolute	AppliChem
Isopropanol	AppliChem
Proteinase K	Sigma-Aldrich
dNTPs	Promega
TE buffer 1X pH 8,0	AppliChem
Material	**Company**
Electrophoresis Power Supply E844	Consort
Fusion FX Imaging System	Vilber
Nanodrop Spectrophotometer ND-1000	Peqlab
Taq Polymerase	Uniklinikum Würzburg
T-Personal Thermocycler	Biometra

To obtain DNA material needed for genotyping, tissue samples from the ears of each mouse were taken. 500 µl lysis buffer and 5 µl proteinase K were added to each sample and put into a water bath at 55 °C overnight. On the next day, samples were centrifuged for 10 min at 10.000 g and 4 °C. The supernatant of every sample was transferred into 500 µl cold isopropanol and inverted gently 5-10 times until a DNA string was visible. Samples were then centrifuged again at 10.000 g and 4 °C for 10 min and the pelleted DNA washed with 500 µl cold ethanol and again centrifuged at 10.000 g and 4 °C for 10 min. Remaining DNA was left to air-dry and 50 µl 1x TE buffer (pH 8.0) were added onto each sample to resuspend the DNA. The samples were then kept on a shaker overnight at 4 °C to ensure a complete DNA resuspension.

In order to determine the *5-Htt* genotype, a PCR was performed. 1 µl of the pure template DNA was mixed with 1 µl of 2,5mM dNTP, primer exon 2 (10 pmol/µl), primer intron 2 (10 pmol/µl) and primer neo new (10 pmol/µl) each. Furthermore, 2,5 µl PCR buffer containing 10 mM $MgCl_2$ and 0.2 µl of Taq DNA polymerase were added. The total volume of 25 µl was filled up with nuclease free water. The PCR-protocol was performed according to the protocol in table 9 below.

Table 9: PCR protocol for *5-Htt* genotyping

Temperature	**Time**	**Repetition**
95°C	3 min	
95°C	45 s	
65,1°C	45 s	35 cycles
72°C	45 s	
72°C	3 min	
10°C	Pause	

The PCR product was visualized using agarose gel electrophoresis. PCR product and a loading dye were separated through gel electrophoresis in a 3% agarose gel at 120 V for 45 min. Visualization of DNA fragments was reached through adding 0.5 µg/ml ethidium bromide to the agarose gel and consequently using an ultraviolet transilluminator. PCR product sizes and primer sequences are shown in table 10 below.

Table 10: Primer sequences and PCR products for *5-Htt* genotyping

Gene	**Primer sequence**
Exon 2	5'-GCG TTT TCC CTA CAT ATG CTA CCA G-3'
Intron 2	5'-AAG CCT CGC ACA GCC TAC CTT AG-3'
Neo-new	5'-CAG CTC ATT CCT CCC ACT CAT GA-3'
PCR-products	Size
Wild type	225bp (exon 2 + intron 2)
5-HTT knockout	272bp (intron 2 + neo new)

2.2.5 Gene expression study

Primers

Primer sequences were chosen using the NCBI BLAST tool. Primers were purchased from Sigma-Aldrich.

Table 11: List of Primers used for the 5-Htt prenatal stress gene expression study

Reference Gene	Full name	Product [bp]	RefSeq.	Primer-Sequence
Gdi2	Rab GDP dissociation inhibitor beta	126	NM_008112	F: GTCAGAATTGGTTGGTTCTGTTC R: AGCTCTTGGATCACACAATCG
Target Gene				
Avp	Vasopressin	125	NM_009732.2	F: CAAGAGGCGGCAAGAGGG R: CACGAAGCAGCCCAGCTC
Avpr1a	Vasopressin receptor 1A	114	NM_016847.2	F: CCTACATCCTCTGCTGGACAC R: CCAGTAACGCCGTGATCGT
Htr1a	Serotonin 1A receptor	144	NM_008308.4	F: GATCTCGCTCACTTGGCTCA R: AAAGCGCCGAAAGTGGAGTA
Htr2a	Serotonin 2A receptor	112	NM_172812.3	F: CCATAGCCGCTTCAACTCCA R: CGAATCATCCTGTAGCCCGA
Htr2c	Serotonin 2C receptor	73	NM_008312	F: GCAATAATGGTGAACCTGGGC R: ACTGCCAAACCAATAGGCCA
Oxt	Oxytocin	141	NM_011025	F: GAGGAGAACTACCTGCCTTCG R: CGAGAAGGCAGACTCAGGGT
Oxtr	Oxytocin receptor	123	NM_001081147	F: ACGTCAATGCGCCCAAAGAA R: GCACGAGTTCGTGGAAGAGAT
Tph2	Tryptophan hydroxylase 2	184	NM_173391	F: TGGGGATTTGATGCCTAGAACC R: TGGGTTCTTTAGAGCATTTTTGTGT

Buffers, solutions and material

Table 12: Buffers, solutions, kits, material and equipment used for the gene expression study

Buffer	**Composition**
TAE buffer	40 mM Tris acetate 1 mM EDTA
Solution	**Company**
2-Mercaptoethanol	Sigma-Aldrich
Chloroform >99% p.a.	CarlRoth
Ethanol absolute	AppliChem
Isopropanol	AppliChem
SYBR Select Mastermix	Applied Biosystems
TE buffer 1X pH 8,0	AppliChem

Kit	Company
AllPrep DNA/RNA/miRNA Universal Kit	Qiagen
5x iScript cDNA synthesis kit	BioRad
RNase free DNase set	Qiagen
Material	**Company**
MaXtract High Density tubes 1,5 ml	Qiagen
Stainless steel beads 5mm	Qiagen
Equipment	**Company**
CFX 1000 Touch 384 cycler	BioRad
NANODROP 8000 Spectrophotometer	Thermo Scientific
TProfessional Basic Thermocycler	Biometra

RNA from brain regions was extracted using an AllPrep DNA/RNA/miRNA Universal Kit (Qiagen). Only minor changes were made following the manufacturers protocol. RNA concentration was measured with a NANODROP 8000 using optical density (OD) at 260nm. To rule out any contamination, OD 260/280 and OD 230/260 were checked.

Real-time quantitative polymerase chain reaction

2µg RNA per sample was used to synthesize cDNA with the iScript™ cDNA synthesis kit. Primer sequences were chosen using the NCBI BLAST tool. Primers were purchased from Sigma-Aldrich. To perform the qPCR reactions, the cDNA stock solution was further diluted using water to a 1:20 working solution. 0.5 µl forward and reverse primer (5 µM) per sample were mixed with 5µl SYBR™ Select Master Mix and 4 µl cDNA to a total volume of 10 µl. A 384-well plate in a CFX384 Real-Time PCR detection system was used for the reactions.

Table 13: protocol for qPCR with 40 cycles

Temperature	Time	Repetition
95°C	2 min	
95°C 60°C	5 sec 30 sec	40x
65°C – 95°C Increments of 0.5°C	5 sec	
4°C	∞	

LinReg PCR program (2017 version, (Ruijter et al. 2009)) was used to calculate mean PCR efficiencies. Results were normalized using Gapdh (Gapdh_3, Qiagen), Gdi2 and Rplp0 (Qiagen). Evaluation of expression, normalization and calculation of relative gene expression results were obtained with qBase+ software (Biogazelle, Ghent, Belgium). Genes were excluded from the analysis, if more than 15% of the samples did not meet the evaluation criteria of less than 0.5 threshold cycles difference between the analyzed triplets.

2.2.6 Statistical analysis

All data were analyzed using SPSS Statistics 26 (IBM Corporation, New York, USA). GraphPad Prism software version 8 (GraphPad Software, La Jolla, California, USA) was used for data graphing. Data sets were visually inspected via boxplots to examine symmetry, skew, variance, and outliers. Additionally, the Levene's test for homogeneity of variances and a Shapiro-Wilk test of normality were performed. Statistical analysis was performed using one-way-ANOVA or two-way-ANOVA. Data are shown as means ± standard errors of the mean (SEM). Differences at trend level are indicated as #$p<0.1$, significant differences as *$p<0.05$, **$p<0.01$, ***$p<0.001$. If necessary, Bonferroni Correction was performed for results significant or at trend level.

2.3 AVP and OXT Immunofluorescence

2.3.1 Animals

Fifteen male and female mice of all three *5-Htt* genotypes aged 4-6 months were sacrificed using isoflurane. Whole dissected brains were then put into 4% paraformaldehyde in PBS for 48 hours, exchanging the solution once after 24h. After washing with PBS 3 times for 15 min, brains were placed 24h in 10% and 20% sucrose solution each, for cryopreservation. Preserved brains were snap-frozen in ice-cold isopentane solution. Brains were sliced into coronal sections of 30µm thickness with a Leica CM1950 freezing microtome, transferred to HistoBond®+ adhesion slides and stored at -80°C degrees Celsius until further use.

2.3.2 Immunofluorescence staining

Slides were left to air-dry for 45 min and subjected to an antigen retrieval using 10 mM Citrate buffer (pH 6.0) at 80°C for 20 min. Slides were then left to cool for 20 min and washed with TBS 3 times for 5 min. Slides were blocked using a blocking solution containing 10% NHS and 0.2% Triton X-100 in TBS for 1h and incubated overnight at 4°C in a wet chamber using primary antibodies rabbit anti-oxytocin (Merck Millipore #AB911, 1:500) and guinea pig anti-vasopressin (Peninsula Laboratories T-5048, 1:100). On the next day, sections were washed with TBS 3 times for 5 min and incubated with corresponding secondary antibodies donkey anti-rabbit IgG Alexa Fluor 488 (Thermo Fisher, 1:400) and donkey anti-guinea pig IgG Alexa Fluor 647 (Thermo Fisher, 1:400) for 1.5 hours. Sections were then washed with TBS and embedded in Fluorogel (Electron Microscopy Sciences) for long-term storage.

2.3.3 Microscopy and image analysis

Fluorescence images were captured with a FluoView F1000 Olympus microscope using a 10X UPlanSAPO (NA 0.75 air) objective. Stacks were taken at illumination wavelengths of 488nm (Alexa Fluor 488) and 635nm (Alexa Fluor 647). Raw images were processed

using the software Fluoview (Olympus). Images were processed using ImageJ software version 1.52n. Analyzed brains were blinded for *5-Htt* genotype and sex. Between 5 and 7 sections per brain were used to analyze the area of the VPN and AVP- and OXT- positive cells. To measure the area of the PVN, division of OXT-positive cells was defined as the PVN, and area borders were measured around the cells. AVP- and OXT-positive cells were counted manually and marked to avoid double counting. Average number of positive cells was calculated for each brain.

2.3.4 Statistical analysis

All data were analyzed using SPSS Statistics 26 (IBM Corporation, New York, USA). GraphPad Prism software version 8 (GraphPad Software, La Jolla, California, USA) was used for data graphing. Data sets were visually inspected via boxplots to examine symmetry, skew, variance, and outliers. Additionally, the Levene's test for homogeneity of variances and a Shapiro-Wilk test of normality were performed. Statistical analysis was performed using one-way ANOVA. Data are shown as means ± standard errors of the mean (SEM). Differences at trend level are indicated as #$p<0.1$, significant differences as *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3 Results

3.1 Effects of Social Fear Conditioning

3.1.1 Social Fear Conditioning affects social behavior

To assess the influence of SFC on social behavior, social investigation time of both SFC+ and SFC- groups was analyzed before and after SFC or social interaction experience. The results of this analysis are represented in figure 9 below.

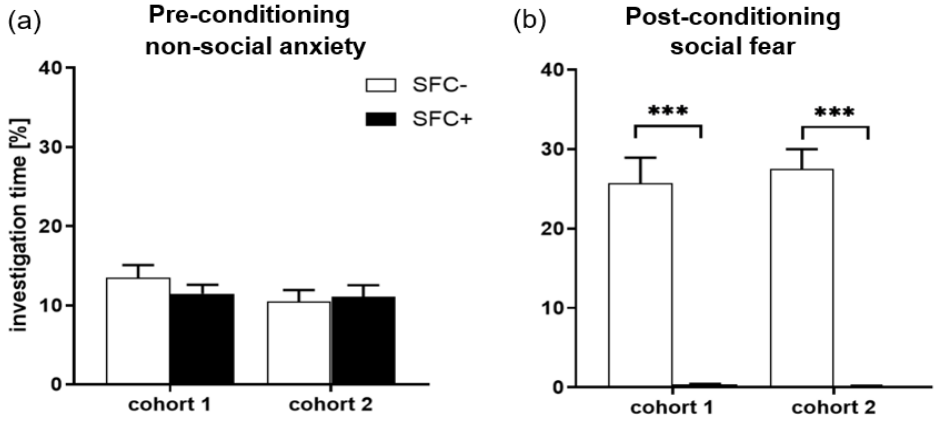

Figure 9: Effects of social fear conditioning on social investigation behavior.
Time spent investigating either an empty wire mesh cage (non-social stimulus) or an unknown conspecific in a wire mesh cage (social stimulus). Cohort 1 and 2 are comprised of 20 animals each, 10 of them SFC+ and 10 SFC-. Animals of cohort 1 were used for the IHC study, animals of cohort 2 were used for the gene expression study. Results are presented as arithmetic means in percent of total investigation time (3 min for SFC-, 5 min for SFC+). A) Investigation time of non-social stimulus pre-SFC, B) investigation time of social stimulus post SFC. Significance levels: p*** <0.001. Adapted from (Hamann et al. 2022).

Investigation time of the non-social stimulus pre-SFC was comparable between both cohorts and treatment groups (Figure 9, a). Investigation time of the social stimulus after SFC was non-existent for the SFC+ groups of both cohorts, whereas the SFC- groups spend a significant amount of time investigating the social stimulus (b). Compared to the non-social stimulus (a), investigation time of the social stimulus (b) in the SFC- groups was a lot longer (10% of the time vs. 25% of the time, approximately). Since the SFC+ groups completely avoided interacting with the social stimulus after SFC experience, the results were satisfactory to the purpose of creating a longer lasting fear of social interaction.

3.1.2 Social Fear Conditioning and corticosterone levels

A different possibility to measure stress-induced changes is to analyze the corticosterone levels, which can be a marker for stress. Corticosterone can be measured within blood plasma samples, which is why we took a blood sample of every mouse within the SFC experiment immediately after they were sacrificed. The result of this analysis can be seen in figure 10. The results failed to reach significance or a trend. The corticosterone concentration of the SFC+ displays a very high SEM, which might be indicative of effects that are not identifiable and might be due to technical differences in the collection method.

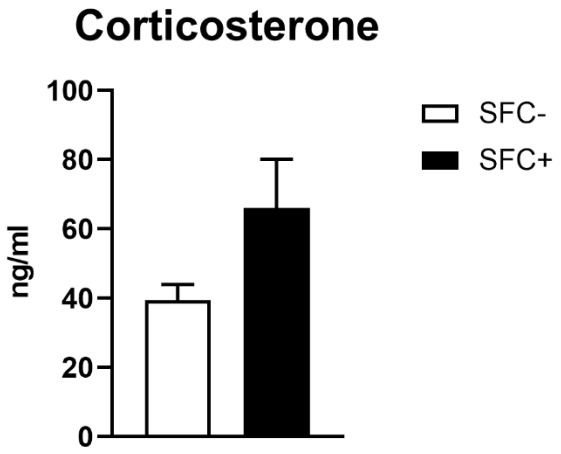

Figure 10: Blood corticosterone concentration after social fear conditioning.
Blood corticosterone levels 2 hours after social fear assessment on day 2 of the SFC paradigm. SFC+, mice exposed to social fear conditioning; SFC-, unconditioned control mice. Data are presented as arithmetic means ± SEM.

3.1.3 Influence of Social Fear Conditioning on cFOS protein expression

The immediate early gene cFos can be used as an example to see alterations in protein expression after stressful experiences. We assessed the effects of SFC on cFOS protein expression in the hippocampal regions DG, CA1 and CA3, which are involved in memory formation and the PVN, as the source of AVP and OXT production. We applied a cFOS-immunolabeling detection method. The hippocampus was divided in two parts, dorsal and ventral. In coronal sections, the border between both parts was defined at the point where the corpus callosum separates and does not connect the two hemispheres anymore (Karabeg et al. 2013) (interaural level 1.34 mm approximately, (Paxinos and Franklin 2004). Paradigmatic immunolabeled sections for the dorsal and ventral region are shown in figures 11 and 12 below, with Nissl staining serving as orientation guide and a naïve brain stained as an example for cFos staining without stimulation. Especially in the CA1 region, the number of cFos-positive cells was very low. A few example cells within each region are magnified.

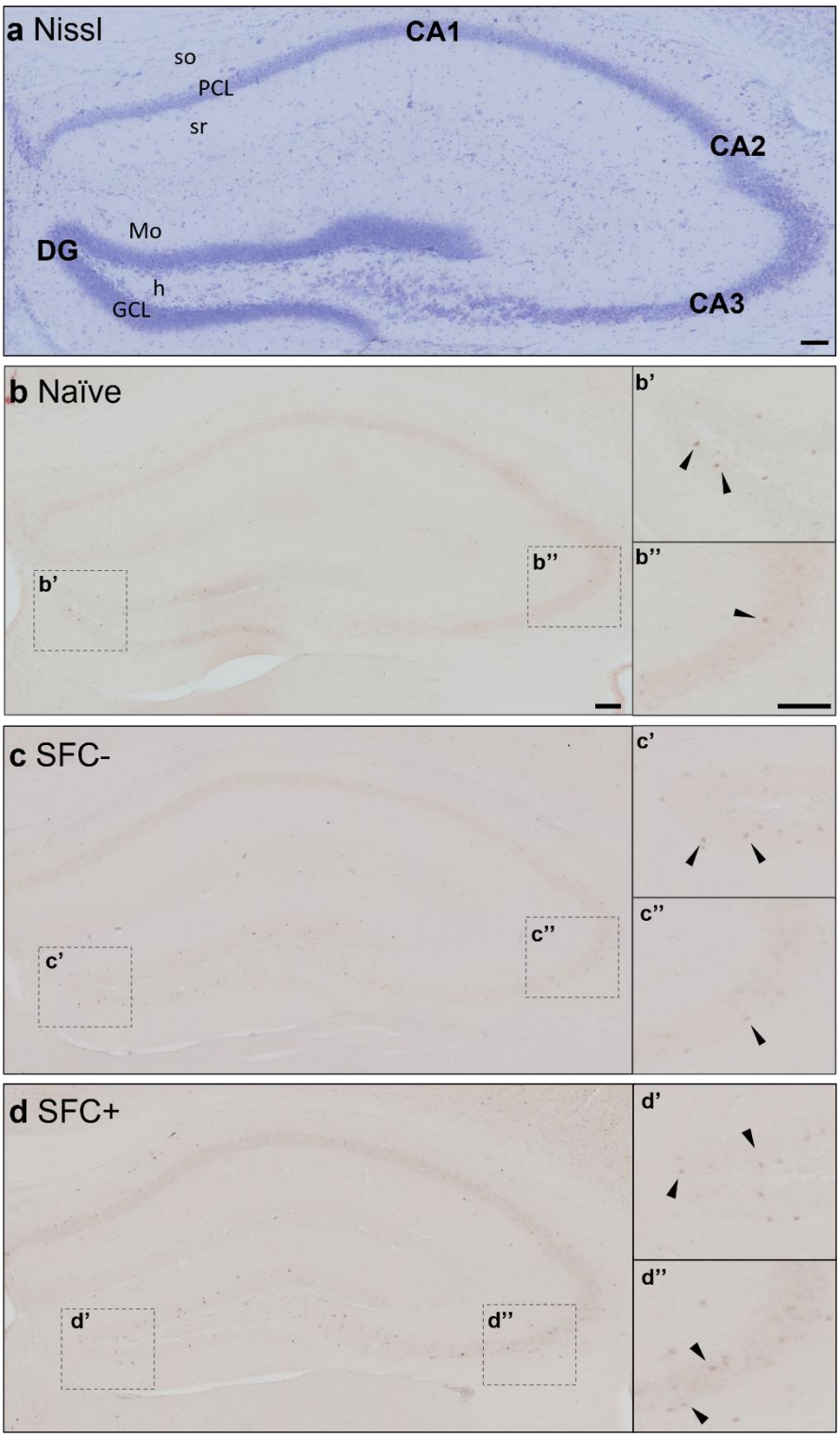

Figure 11: Representative images of immunohistochemical stainings of cFOS-positive cells in the dorsal hippocampus.

a: Nissl staining showcasing the parts of the hippocampus used for the analysis. b: a naïve control hippocampus without social contact. Magnification of the DG (b') and the CA3 (b''). c: example of an animal belonging to the SFC- group. Magnification of the DG (c') and the CA3 (c''). d: example of an animal belonging to the SFC+ group. Magnification of the DG (d') and the CA3 (d''). Arrowheads are pointing towards cFos-positive cells. gcl = granular cell layer, h = hilus, Mo = Molecular Layer, so = stratum oriens, sr = stratum radiatum. Scale bar = 100µm. These images were created together with master student J. Bankmann and are adapted from his master thesis (Bankmann, 2021).

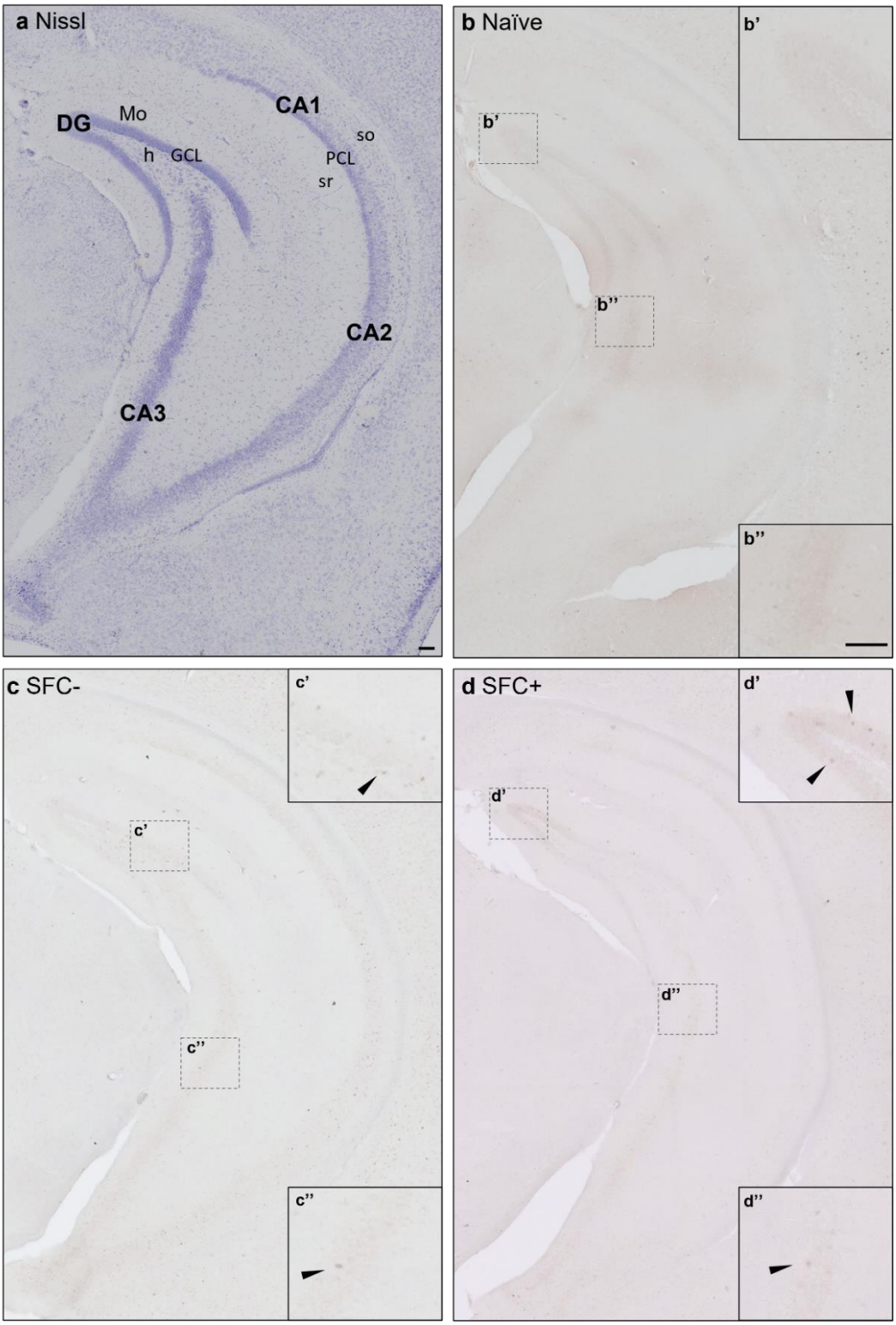

Figure 12: Representative images of immunohistochemical stainings of cFOS-positive cells in the ventral hippocampus.

a: Nissl staining showcasing the parts of the hippocampus used for the analysis. b: a naïve control hippocampus without social contact. Magnification of the DG (b') and the CA3 (b''). c: example of an animal belonging to the SFC- group. Magnification of the DG (c') and the CA3 (c''). d: example of an animal belonging to the SFC+ group. Magnification of the DG (d') and the CA3 (d''). Arrowheads are pointing towards cFOS-positive cells. gcl = granular cell layer, h = hilus, Mo = Molecular Layer, so = stratum oriens, sr = stratum radiatum. Scale bar = 100µm. These images were created together with master student J. Bankmann and are adapted from his master thesis (Bankmann, 2021).

Evaluation of the cFOS-positive cells was achieved through manual counting using the Stereo Investigator software as an unbiased stereology system. Results for the DG, CA3 and CA1 region are shown in figure 13.

Statistical evaluation revealed no differences between SFC- and SFC+ groups in the ventral hippocampus of all three evaluated regions. Mean number of cFOS-positive cells, as well as the mean cell density in the dorsal part of the DG was significantly higher in the SFC- group compared to the SFC+ group (figure 13 c, p=0.004 for both). CA3 and CA1 regions of the dorsal hippocampus showed no significant differences or differences on trend level.

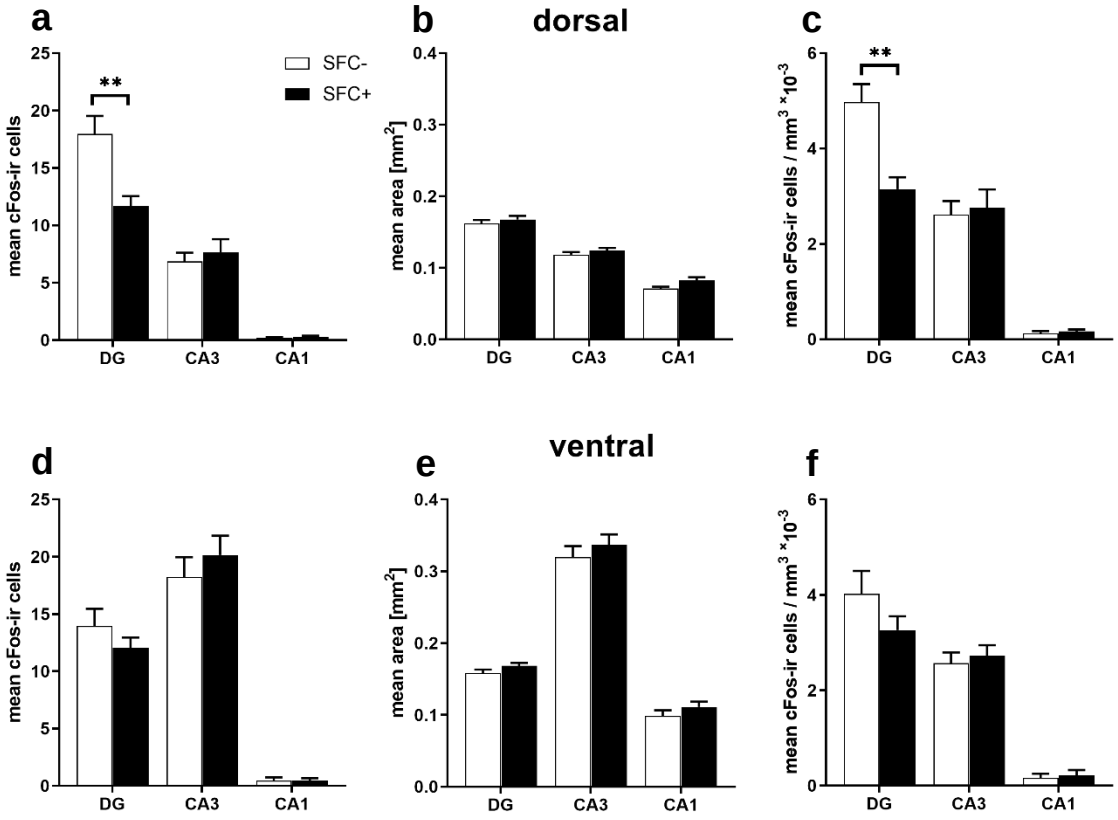

Figure 13: Quantitative evaluation of cFOS-immunoreactive cells in the dorsal and ventral hippocampus.
Arithmetic means of cFOS-immunoreactive (ir) cells in dorsal (a) and ventral (d) hippocampus of dentate gyrus (DG) granular cell layer and pyramidal cell layers of cornu ammonis area 3 (CA3) and 1 (CA1). Mean area of investigated layers in mm^2 (b, e). Mean cell density (mean number of cFOS-ir cells/mm^3; c, f). SFC+, mice exposed to social fear conditioning, SFC-, unconditioned control mice. Statistical analysis was performed using a two-sample t-test and Bonferroni post-hoc test, if applicable; n=10 for both groups. Data are presented as arithmetic means ± SEM. p**<0.01. Adapted from (Hamann et al. 2022).

In addition to the hippocampus region, we also wanted to investigate the PVN to look for possible connections between SFC treatment and the AVP and OXT brain systems. Since the borders of the PVN are not easily distinguishable through light microscopical analysis, we opted to analyze cFOS protein expression with the help of a counterstaining with AVP.

The area of AVP positive cells helps to identify the borders of the PVN. To facilitate the staining process, we opted for an immunofluorescence approach. An example staining is shown in figure 14 below. The PVN area was measured around the AVP-positive cells. CFOS-positive cells which are outside of the area of AVP-positive cells and fibers, were

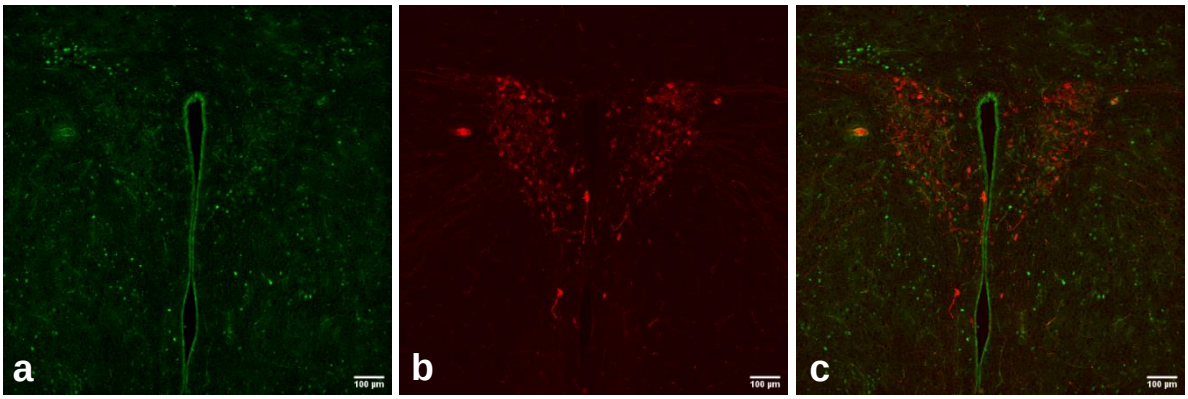

Figure 14: Representative images of an immunofluorescence double staining of cFOS and AVP in the PVN.
Immunofluorescence staining of coronal sections of an SFC- animal with antibodies detecting cFOS and AVP. a: cFOS (green); b: AVP (red), c: overlay of both images. Scale bar = 100µm.

therefore excluded from the analysis. Double-positive cells were few and scarcely distributed.

Quantitative evaluation of the cFOS-staining was done by manual counting of cFOS-positive cells in the PVN area. Imaging was performed using an Olympus FluoView FV1000 Confocal microscope with a 10x UPlanAPO 0.40 NA objective. Raw images were processed with the internal FluoView imaging software. The distribution of cFOS-positive cells was widespread along the brain, not necessarily limited to a specific area. The results of the cell-counting are shown in figure 15. There were no significant differences or differences on trend level between SFC+ and SFC- groups.

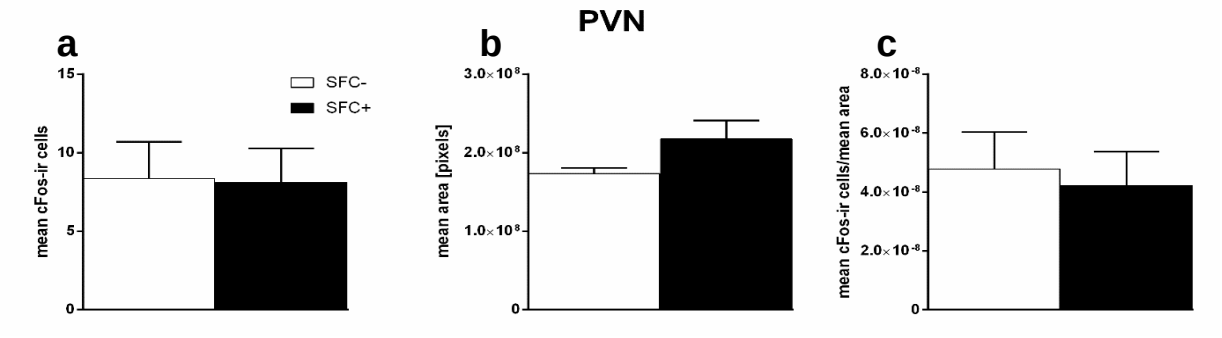

Figure 15: Quantitative evaluation of cFos-immunoreactive cells in the PVN of the hypothalamus.
Arithmetic means of cFOS immunoreactive (ir) cells in the PVN of the hypothalamus (a). Mean area of investigated layers in pixels (b,). Mean cell density (mean number of cFOS-ir cells/pixels; c). SFC+, mice exposed to social fear conditioning, SFC-, unconditioned control mice. Statistical analysis was performed using a two-sample t-test and Bonferroni post-hoc test, if applicable; n=10 for both groups. Data are presented as arithmetic means ± SEM. Adapted from (Hamann et al. 2022).

3.1.4 Influence of Social Fear Conditioning on gene expression

Protein expression is usually detectable at later time points, which makes it possible that changes on protein levels are masked if the time point of scarification is too early after stressful exposure. In order to catch alterations that have not reached the protein level yet, we performed a gene expression analysis. Due to time capacities, only the dorsal part of the hippocampus was used for the evaluation using a laser capture microdissection microscope. Evaluation of immediate early genes *Arc*, *cFos* and *Fosl2* was performed in the septum, hippocampal subregions DG, CA3, CA1 and PVN, BLA and DR (figure 16). This revealed significantly lower expression of *cFos* and *Fosl2* in the CA3 region (figure 16 b, p=0.006 and p=0.037) of SFC+ animals, with c*Fos* showing a strong expression difference. Additionally, we found a significantly lower expression of *Fosl2* in the CA1 region (figure 16 c, p=0.032). No significant differences or differences on trend level were found in the other investigated regions.

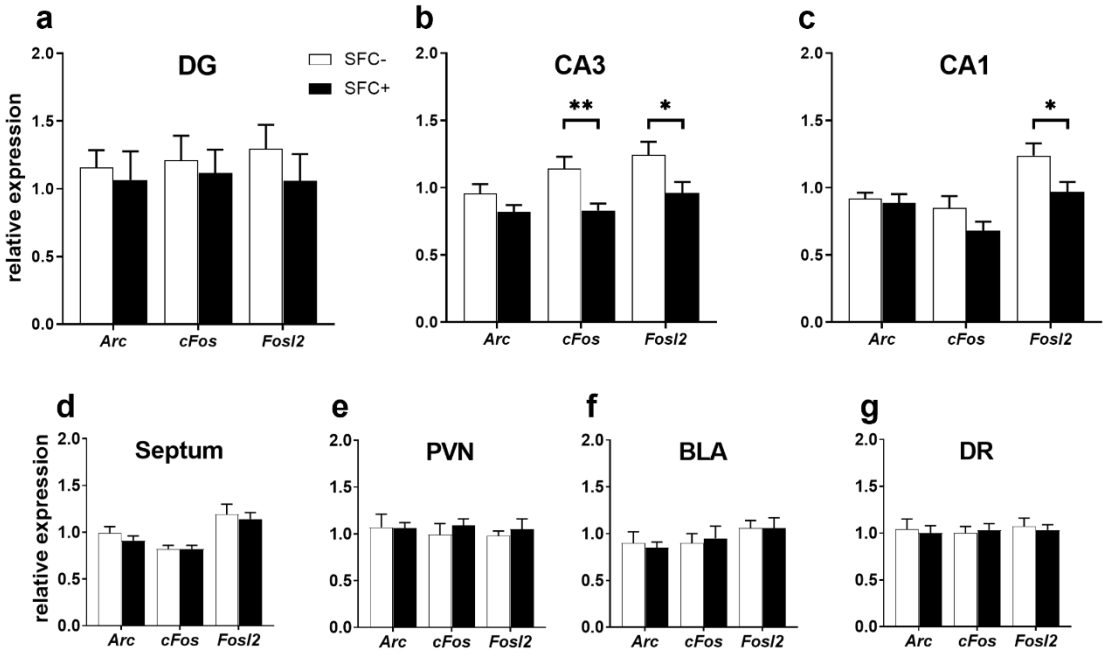

Figure 16: Social Fear Conditioning alters gene expression of immediate early genes in hippocampal regions.
Relative gene expression of immediate early genes arc, cFos and Fosl2 in dentate gyrus (DG, a), cornu ammonis area 3 (CA3, b), cornu ammonis area 1 (CA1, c), septum (d), paraventricular nucleus (PVN, e), basolateral amygdala (BLA, f), dorsal raphe (DR, g). SFC+; mice exposed to social fear conditioning; SFC- unconditioned control mice. Statistical analysis was performed using a two-sample t-test and Bonferroni post-hoc test, if applicable; n=10 for both groups. Data are presented as arithmetic means ±SEM. p**<0.01, p*<0.05, p#<0.1. Adapted from (Hamann et al. 2022).

In a next step, genes of the NPY system were investigated, again in the aforementioned regions. The three investigated genes were *Npy* and its receptors *Npyr1* and *Npyr2* (figure 17). Evaluation showed significantly higher expression of *Npyr2* in the septum of SFC+ animals (figure 17 d, p=0.035). Trending higher expressions were found for *Npyr1* in the PVN of SFC+ animals (figure 17 e, p=0.064), *Npyr1* and *Npyr2* in the BLA of SFC+ animals (figure 17 f, p=0.069 and p=0.074) and *Npy* (figure 17 g, p=0.077) in the DR of SFC-

animals. No significant differences or differences at trend level were found in the hippocampal regions DG, CA3 and CA1.

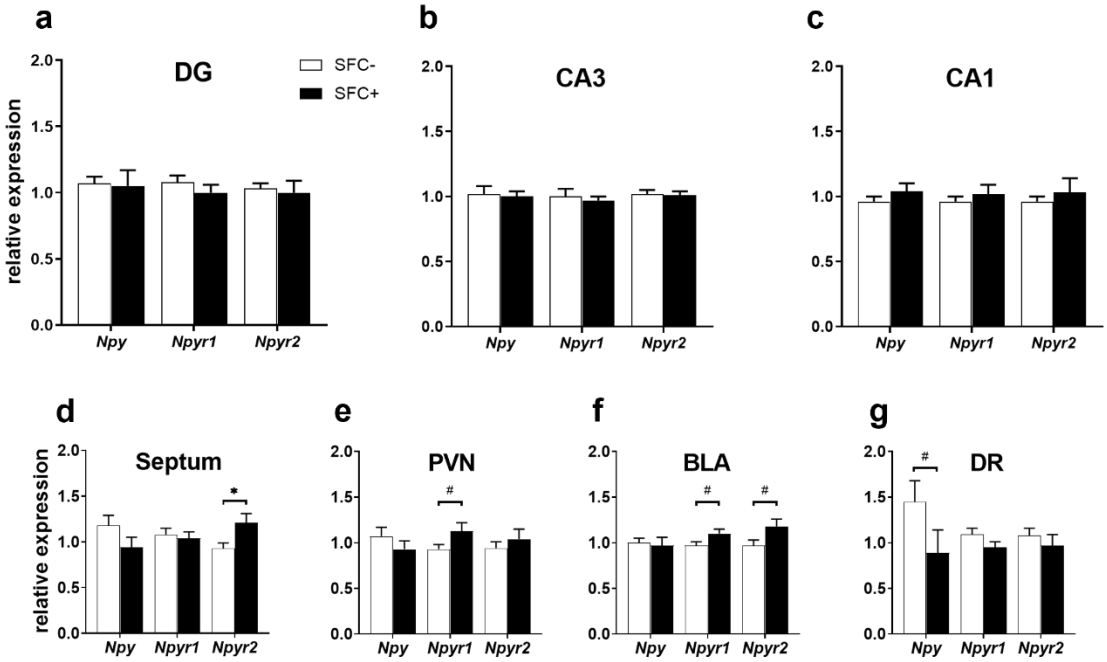

Figure 17: Social Fear Conditioning exclusively affects gene expression of neuropeptide Y receptor 2 in the septum.
Relative gene expression of neuropeptide Y (Npy) and its receptors neuropeptide 1(Npyr1) and neuropeptide receptor 2 Npyr2) in dentate gyrus (DG, a), cornu ammonis area 3 (CA3, b), cornu ammonis area 1 (CA1, c), septum (d), paraventricular nucleus (PVN, e), basolateral amygdala (BLA, f), dorsal raphe (DR, g). SFC+; mice exposed to social fear conditioning; SFC- unconditioned control mice. Statistical analysis was performed using a two-sample t-test and Bonferroni post-hoc test, if applicable; n=10 for both groups. Data are presented as arithmetic means ±SEM. p*<0.05, p#<0.1. Adapted from (Hamann et al. 2022).

To evaluate the serotonin system, we decided to narrow the analysis down to the two serotonin receptors *Htr1a* and *Htr2a.* Again, investigating the already established regions, we were able to show a significantly higher gene expression of *Htr2a* in the PVN of the SFC+ group compared to the SFC- group (figure 18 e, p=0.023). The other investigated regions did not show any significant changes or changes at trend level in gene expression of the two receptors. Interestingly, relative expression of *Htr1a* in the BLA was overall a lot weaker than all other investigated regions.

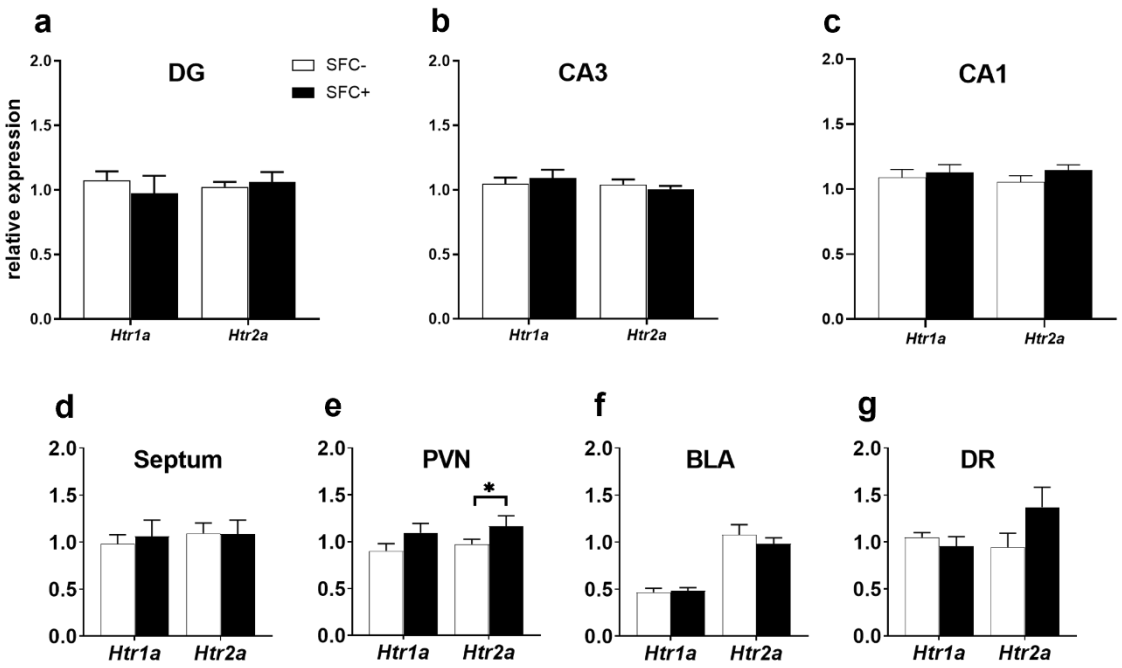

Figure 18: Social Fear conditioning exclusively affects serotonin receptor 2a in the PVN. Relative gene expression of serotonin receptor 1a (Htr1a) and serotonin receptor 2a (Htr2a) in dentate gyrus (DG, a), cornu ammonis area 3 (CA3, b), cornu ammonis area 1 (CA1, c), septum (d), paraventricular nucleus (PVN, e), basolateral amygdala (BLA, f), dorsal raphe (DR, g). SFC+; mice exposed to social fear conditioning; SFC- unconditioned control mice. Statistical analysis was performed using a two-sample t-test and Bonferroni post-hoc test, if applicable; n=10 for both groups Data are presented as arithmetic means ±SEM. p*<0.05. Adapted from (Hamann et al. 2022).

For the AVP and OXT brain systems we decided to limit our analysis to the two receptors *Avpr1a* and *Oxtr*, which allowed investigation in all regions. Results of this analysis are shown in figure 19. We could not detect any significant differences or differences at trend level in the investigated regions. The result for *Avpr1a* in the CA3 and BLA region had to be discarded from the analysis due to the fact that too many samples had to be excluded from evaluation of the qPCR.

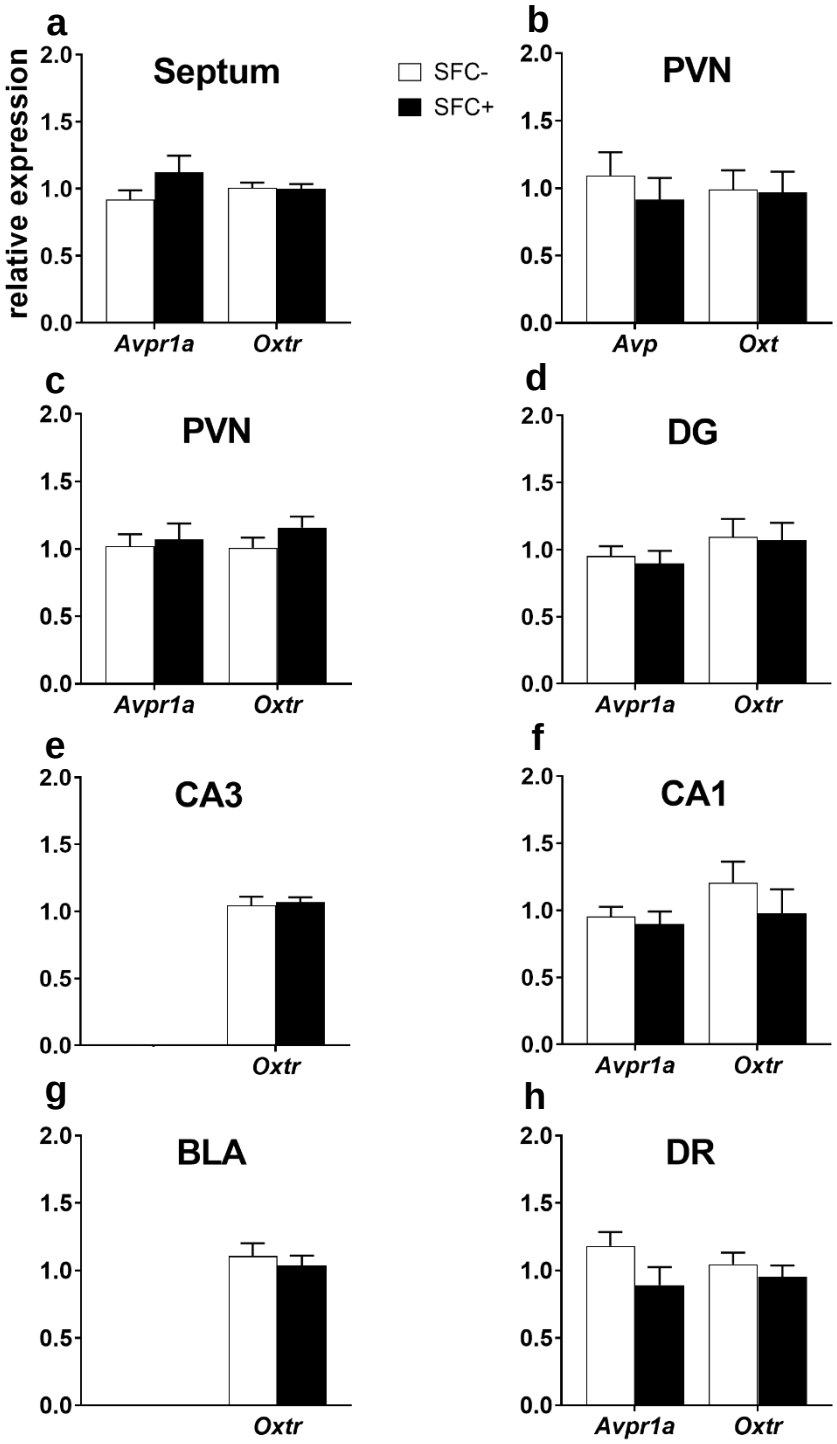

Figure 19: Social Fear Conditioning has no effects on gene expression of vasopressin and oxytocin and their respective receptors.
Relative gene expression of oxytocin receptor (Oxtr) in septum (a), paraventricular nucleus (c), dentate gyrus (d), cornu ammonis area 3 (CA3, e), cornu ammonis area 1 (CA1, f), basolateral amygdala (g), dorsal raphe (h). Vasopressin receptor (Avpr1a) gene expression in a, c-f, h Vasopressin (Avp) and Oxytocin (Oxt) expression in the PVN (b). SFC+; mice exposed to social fear conditioning; SFC- unconditioned control mice. Statistical analysis was performed using a two-sample t-test and Bonferroni post-hoc test, if applicable; n=10 for both groups. Data are presented as arithmetic means ±SEM.

The results of all statistical analyses of the SFC-study gene expression are presented in table 14 of the appendix.

3.2 Influence of prenatal stress and serotonin transporter deficiency on gene expression

Prenatal stress and 5-HTT deficiency affect gene expression in female offspring

Stressful events can not only influence the individual directly affected by it, but pregnant humans, as well as animals can pass negative effects of different stressors on to the unborn child (in case of humans) or litter (in case of mice). It is known that stressful events during pregnancy, as well as two short alleles of the human *5-HTT* gene can lead to a higher risk to develop anxiety disorders in the future. In mice, reduced 5-HTT levels are associated with elevated anxiety behavior and higher levels of general anxiety. 5-HTT KO mice are an established model to study anxiety disorders. Since it is not known, which processes on the molecular level facilitate the development of anxiety disorders, a prenatal stress paradigm was applied to pregnant 5-HTT WT dams that had been bred together with male 5-HTT HET mice. During late stages of the pregnancy, dams were exposed to the prenatal stress paradigm. Due to the breeding scheme, genotypes of offspring animals were limited to WT and HET. After weaning, female offspring was subjected to behavioral testing in order to assess general anxiety like- and their social behavior. The prenatal stress paradigm, as well as the behavioral tests of the offspring was performed by Karla Schraut and is part of her PhD-thesis (Schraut 2015).
We investigated the expression of genes of the social neuropeptides AVP and OXT as well as their respective receptors AVPR1A and OXTR. We decided to analyze different brain regions that are either relevant to the AVP and OXT systems (hypothalamus), important for learning processes (hippocampus), involved in emotional regulation (amygdala), or the source of serotonin production (raphe). Due to the indication of AVP-producing neurons in the hippocampus, we decided to look investigate AVP gene expression in this region as well. Gene expression was analyzed via two-way ANOVA, evaluating genotype and treatment as factors. Levene's test for homogeneity of variances and Shapiro-Wilk test for normal distribution were also performed.
Looking at the AVP system (figure 20), the analysis revealed significant differences for *Avpr1a* in the amygdala, with both the PS groups showing significantly higher expression than the control groups (figure 20 c, p=0.046) as well as WT animals showing significantly higher gene expression than HET animals (figure 20 c, p=0.040). The raphe region also showed significantly higher expression of *Avpr1a* in the PS group compared to the control group (figure 20 d, p=0.025). A rather uncommon result could be observed in the hippocampus, where the HET control group showed significantly higher *Avp* gene expression than all other groups, which, together with the other results, led to significant differences for all investigated parameters (figure 20 b, p=0.045 for genotype, p=0.034

for treatment, p=0.002 for interaction between genotype and treatment). No significant differences or differences at trend level could be found in the hypothalamus.

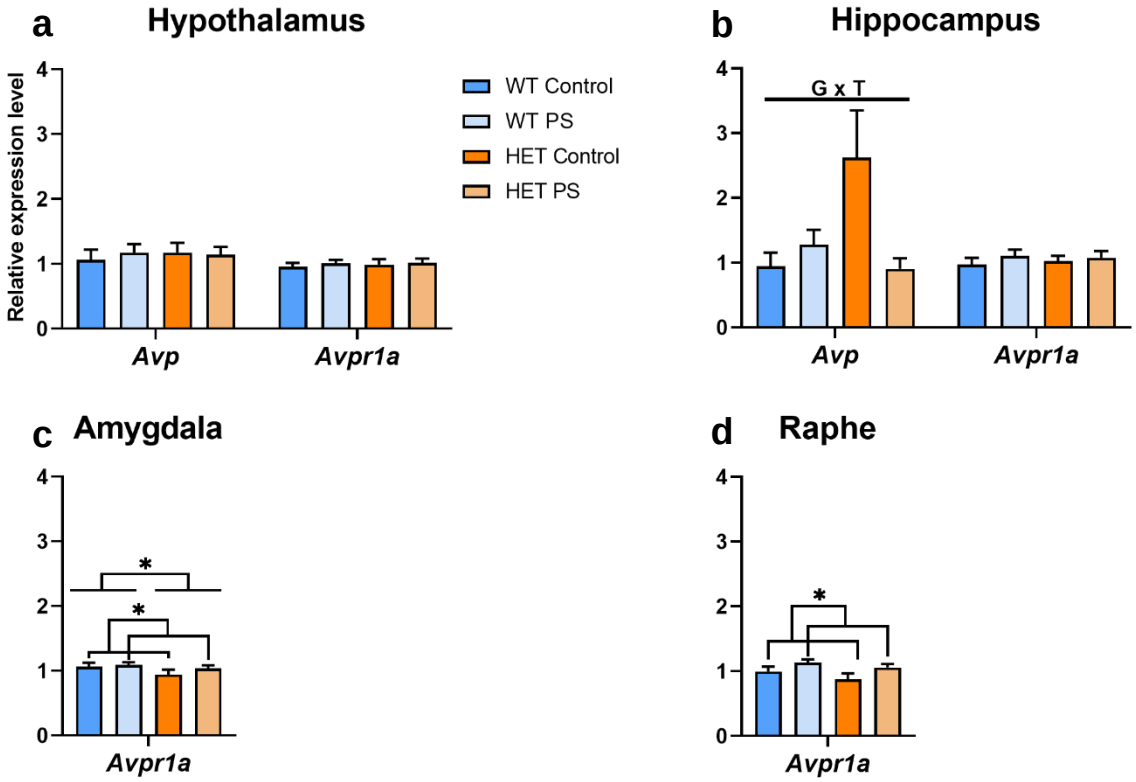

Figure 20: Influence of prenatal stress on genes belonging to the AVP system in female mice of two different *5-Htt* genotypes.
Relative expression of the *Avpr1a* genes in hypothalamus (a), hippocampus (b), amygdala (c) and raphe (d). Relative gene expression of *Avp* (a). WT: wild type animals; HET: 5-HTT heterozygous mice; C: control animals without prenatal stress exposure; PS: animals with prenatal stress exposure. Statistical analysis was performed using two-way ANOVA, Levene's test and Shapiro-Wilk test (WT Control, n=12; WT PS, n=23; HET Control, n=11; HET PS, n=27). Data are presented as arithmetic means ±SEM. p*<0.05. GxT: significant gene x treatment interaction.

Investigation of the *Oxt* system (figure 21) revealed a significant treatment difference within the hippocampus, where *Oxtr* expression of PS animals was significantly higher than control animals (figure 21 b, p=0.043)). A trend could be found for the same constellation in the raphe (figure 21 d, p=0.052). No significant differences or differences at trend level were detected in the other investigated regions.

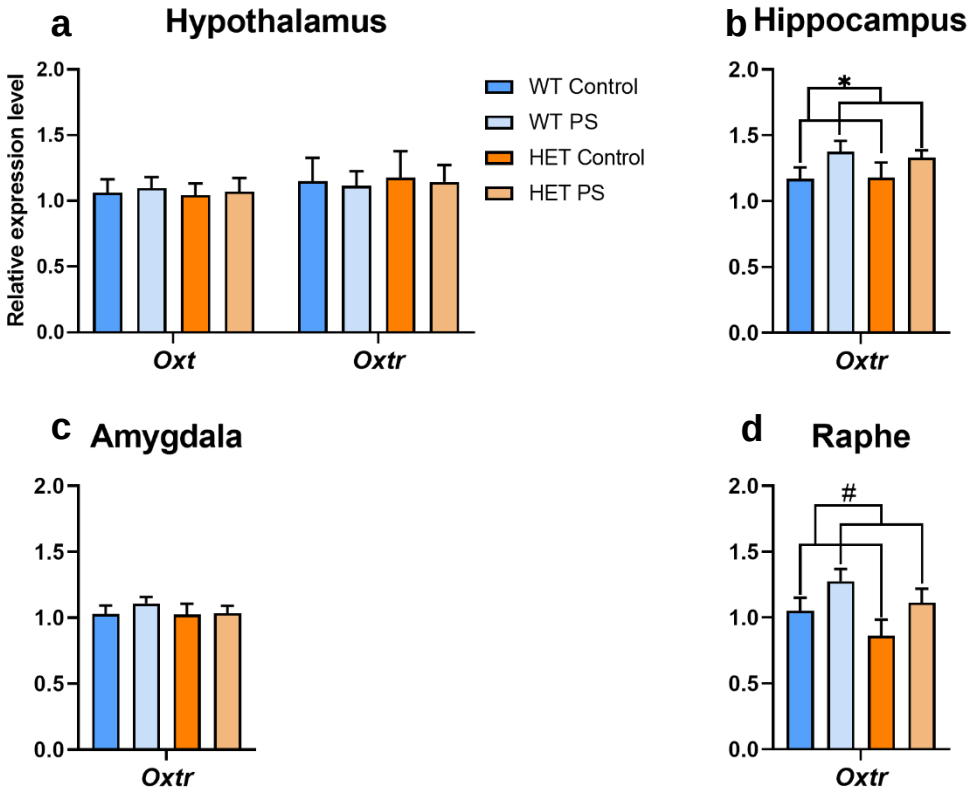

Figure 21: Influence of prenatal stress on the expression of genes related to the OXT system in female mice of two different *5-Htt* genotypes.
Relative expression of the *Oxtr* gene in hypothalamus (a), hippocampus (b), amygdala (c) and raphe (d). Relative gene expression of *Oxt* (a). WT: wild type animals; HET: 5-HTT heterozygous mice; C: control animals without prenatal stress exposure; PS: animals with prenatal stress exposure. Statistical analysis was performed using two-way ANOVA, Levene's test and Shapiro-Wilk test (WT Control, n=12; WT PS, n=23; HET Control, n=11; HET PS, n=27). Data are presented as arithmetic means ±SEM. p*<0.05, p#<0.1.

Looking at the serotonergic system revealed results at trend level within the raphe region for both the expression of *Htr1a* and *Htr2a* receptors. Gene expression was lower in HET animals compared to WT animals (figure 22 d, p=0.059 for *Htr1a*, p=0.085 for *Htr2a*). Additionally, *Htr1a* showed a trend of higher expression within the PS groups compared to the control groups (figure 22 d, p=0.069). Gene expression results in the other three regions revealed no significant differences or trends. Investigation of *Tph2* in the raphe however, revealed significantly higher gene expression in WT animals compared to HET animals (figure 22 e, p=0.003).

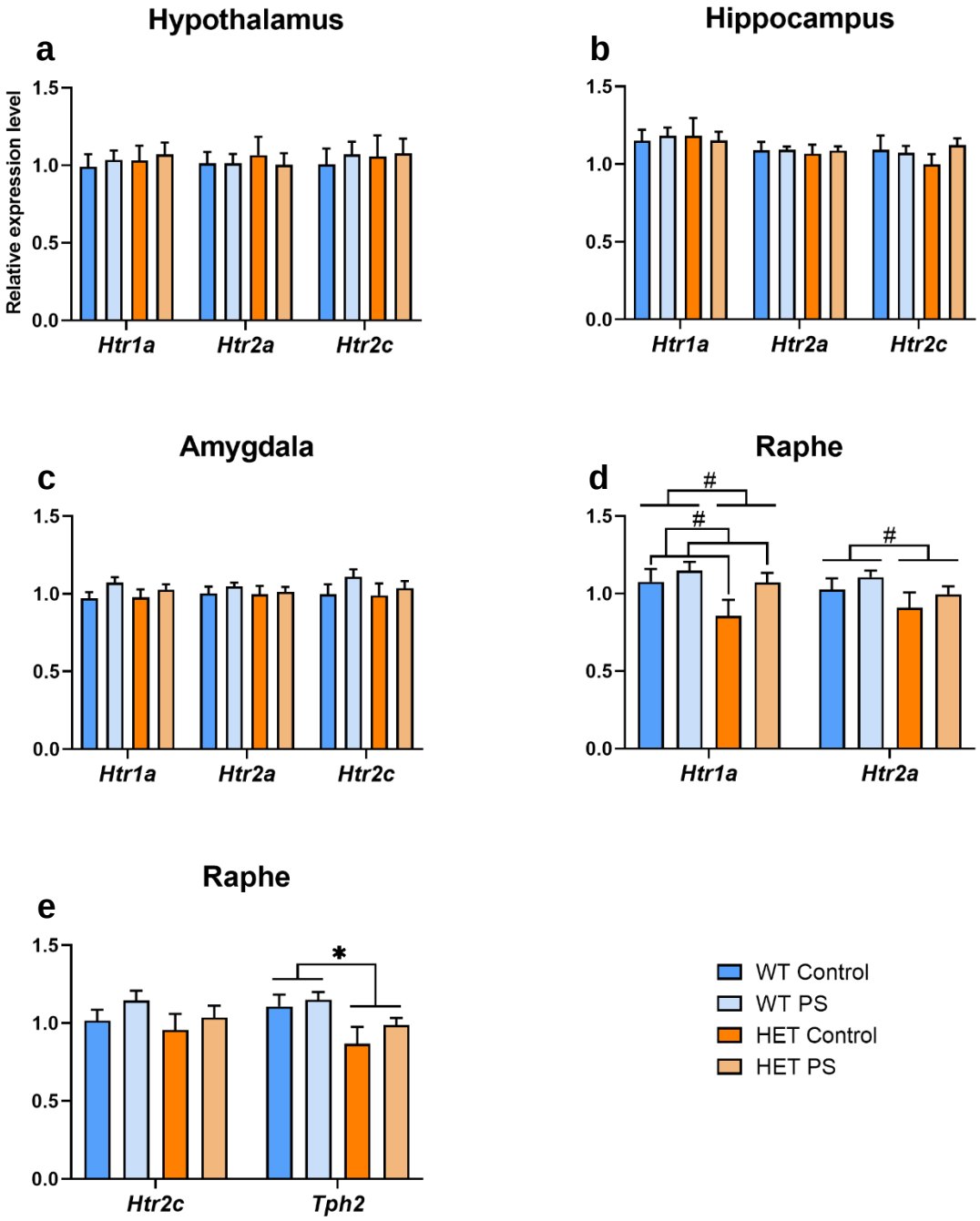

Figure 22: Influence of prenatal stress on serotonin receptor gene expression in female mice of two different *5-Htt* genotypes.
Relative gene expression of serotonin receptors *Htr1a*, *Htr2a* and *Htr2c* in hypothalamus (a), amygdala (b), hippocampus (c) and raphe (d, e). Relative gene expression of *Tph2* in the raphe (e). WT wild type animals, HET, 5-HTT heterozygous mice; C control animals without prenatal stress exposure, PS animals with prenatal stress exposure. Statistical analysis was performed using two-way ANOVA, Levene's test and Shapiro-Wilk test (WT Control, n=12; WT PS, n=23; HET Control n=11; HET PS, n=27). Data are presented as arithmetic means ±SEM, p*<0.05).

All results of the statistical analysis for each group of this analysis are presented in table 15 of the appendix.

A part of the prenatally stressed animals showed noticeably low levels of social investigation behavior in the 3-chamber sociability test. This resulted in a division of the prenatal stress group into the two groups “social” and “unsocial”, based on the results of the 3-chamber sociability test (for details see (Schraut 2015)). In order to investigate changes on a genetic level that might be contributing to the “unsocial” behavior, we repeated our two-way ANOVA analysis on the basis of this diverging classification. The results showed differences at trend level for the expression of *Avpr1a* in the amygdala between genotypes WT and HET (figure 23 d, p=0.075), with slightly higher expression in the WT. Likewise, *Avpr1* had a slightly higher expression in the PS unsocial group compared to the control group, independent from *5-Htt* genotype (e, p=0.066). Results of A*vpr1a* expression in the other regions, as well as *Oxtr* receptors in all investigated regions showed no significant or trending differences. Expression of *Avp* and *Oxt* in the hypothalamus also did not show any differences.

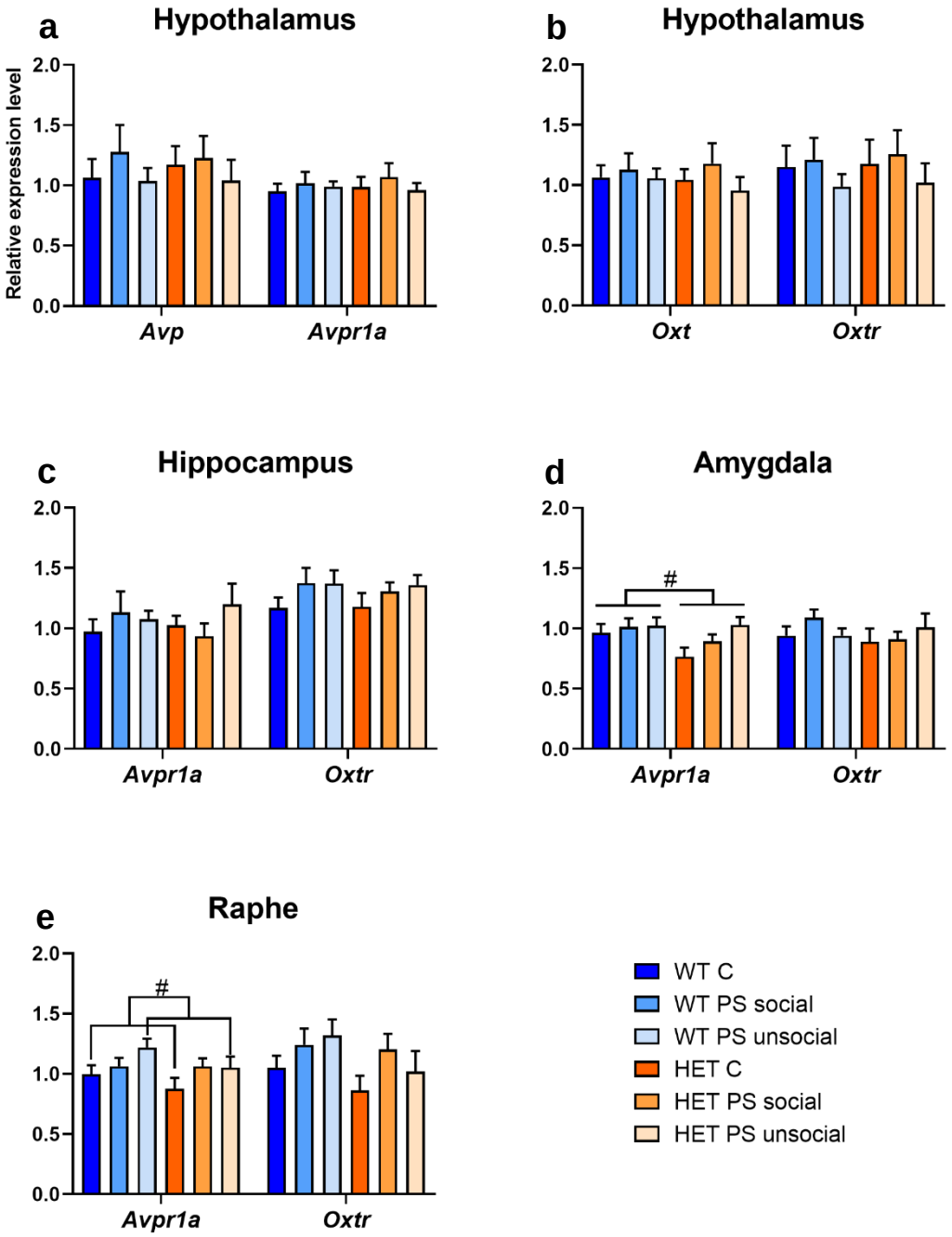

Figure 23: Influence of prenatal stress on Avp and Oxt gene expression in the brain of female mice of two different *5-Htt* genotypes - with or without reduced social behavior.
Relative gene expression of *Avpr1a* and *Oxtr* in hypothalamus (a.b), amygdala (c), hippocampus (d) and raphe (e). Relative gene expression of *Avp* and *Oxt* in the hypothalamus (a,b). WT: wild type animals, HET: mice heterozygous for the 5-Htt knockout; C: control animals without prenatal stress exposure; PS: animals with prenatal stress exposure; social: animals with higher levels of social investigation behavior in the 3-chamber sociability test; unsocial: animals with reduced social investigation behavior in the 3-chamber sociability test. Statistical analysis was performed using two-way ANOVA, Levene's test, Shapiro-Wilk test and Bonferroni post-hoc test, if applicable (WT C, n=10; WT PS social, n=13; WT PS unsocial, n=10; HET C, n=11; HET PS social, n=14; HET PS unsocial, n=13). Data are presented as arithmetic means ±SEM with p#<0.1.

In the same analysis, we also looked into the expression of genes belonging to the serotonergic system and its receptors, to see if the prenatal stress together with alterations of the 5-HT homeostasis affected other levels of the system. The results showed the trend of a genotype effect for the serotonin receptors *Htr1a* and *Htr2a* in the raphe (Figure 24 d, p= 0.082 for *Htr1a* and p=0.066 for *Htr2a*). In addition to that, expression of *Tph2* in the raphe, the limiting enzyme for serotonin synthesis, is significantly higher in WT animals than in HET animals (figure 25 d, p=0.003). The other investigated regions did not show any significant differences for the three investigated serotonin receptors.

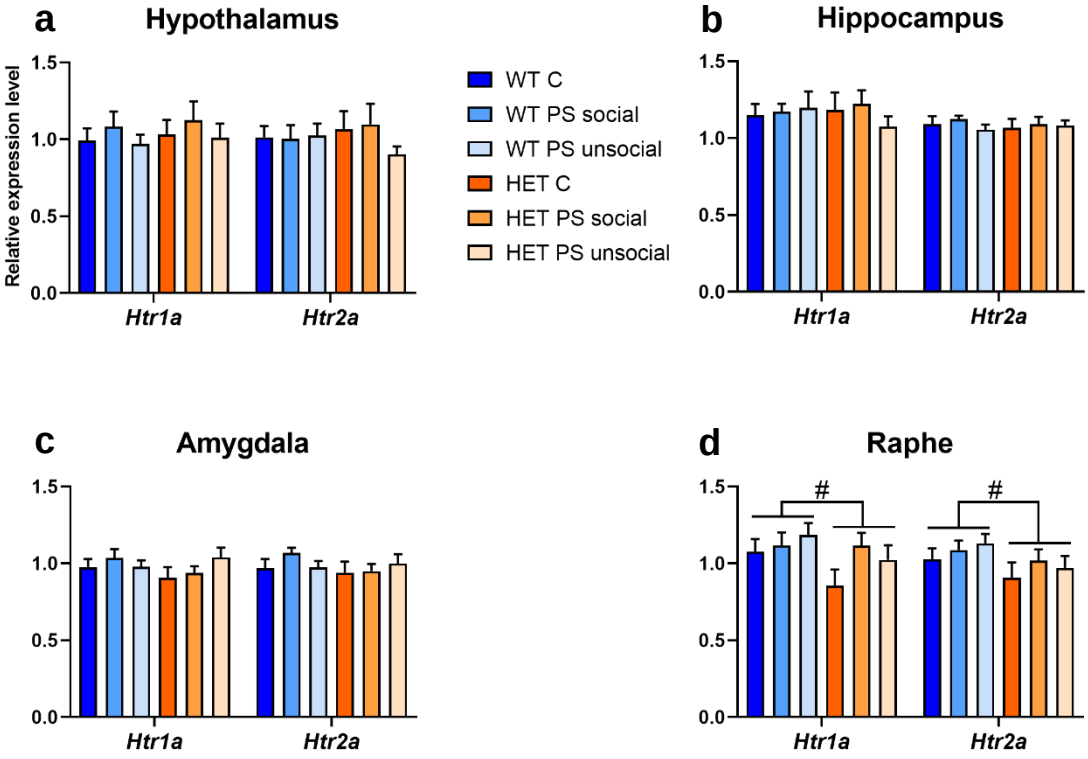

Figure 24: Influence of prenatal stress on serotonin receptor 1a and 2a gene expression in female mice of both *5-Htt* genotypes - with normal or reduced social behavior.
Relative expression of the serotonin receptors Htr1a and Htr2a genes in hypothalamus (a), hippocampus (b), amygdala (c) and raphe (d). WT: wild type animals; HET: mice heterozygous for the *5-Htt* knockout; C: control animals without prenatal stress exposure; PS: animals with prenatal stress exposure; social: animals with higher levels of social investigation behavior in the 3-chamber sociability test; unsocial: animals with reduced social investigation behavior in the 3-chamber sociability test. Statistical analysis was performed using two-way ANOVA, Levene's test, Shapiro-Wilk test and Bonferroni post-hoc test, if applicable (WT C, n=10 (hippocampus n=12); WT PS social, n=13; WT PS unsocial, n=10; HET C, n=11; HET PS social, n=14; HET PS unsocial, n=13). Data are presented as arithmetic means ±SEM.

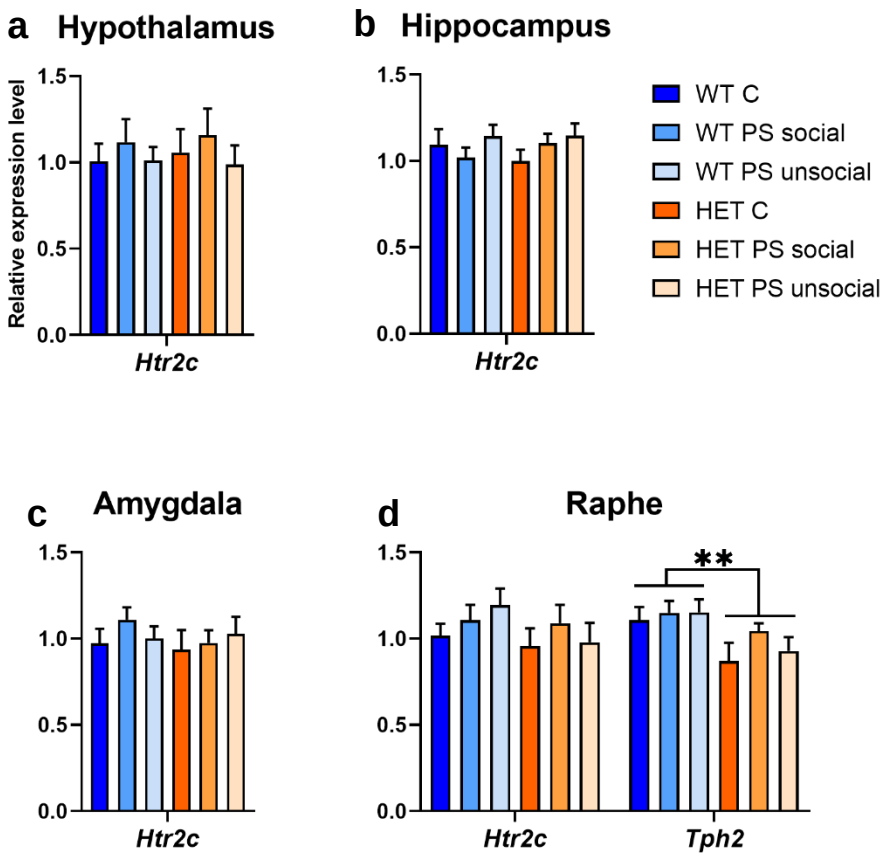

Figure 25: Influence of prenatal stress on the expression of genes belonging to the serotonergic system in female mice of both *5-Htt* genotypes - with normal or reduced social behavior.

Relative gene expression of serotonin receptor Htr2c in hypothalamus (a), amygdala (b), hippocampus (c) and raphe (d). Relative gene expression of Tph2 in the raphe (d). WT: wild type animals; HET: mice heterozygous for the *5-Htt* knockout; C: control animals without prenatal stress exposure; PS: animals with prenatal stress exposure; social: animals with higher levels of social investigation behavior in the 3-chamber sociability test; unsocial: animals with reduced social investigation behavior in the 3-chamber sociability test. Statistical analysis was performed using two-way ANOVA, Levene's test, Shapiro-Wilk test, and Bonferroni post-hoc test, if applicable (WT C, n=10 (hippocampus n=12); WT PS social, n=13; WT PS unsocial, n=10; HET C, n=11; HET PS social, n=14; HET PS unsocial, n=13). Data are presented as arithmetic means ±SEM. p**<0.01.

All results of the statistical analysis for each group of this analysis are presented in table 16 of the appendix.

3.2.1 Prenatal stress and 5-HTT deficiency affect gene expression in male offspring

As a next step, we decided to investigate the male cohorts of the 5-HTT prenatal stress paradigm that had been left without behavioral testing after birth. Due to the breeding scheme, only WT and HET genotypes were possible for the offspring. Since the unborn animals were still exposed to the prenatal stress paradigm, we wanted to see the influence this possibly had on brain systems involved in modulating behavior, on a molecular level. In addition to that, it would be interesting to see whether the results differ from the female counterpart. It is known that neurotransmitter systems which regulate social behavior can work very differently in males and females. We again chose to investigate hypothalamus, hippocampus, amygdala and raphe, to keep results comparable. Additionally, we analyzed the *Npy* system, to see if there might be parallels to the results of our social fear conditioning paradigm in male mice. Gene expression was again analyzed via two-way ANOVA, with genotype and treatment as fixed factors. Levene's test for homogeneity of variances and Shapiro-Wilk test for normal distribution were also performed. Results of our qPCR study analyzing the *Avp* and *Oxt* system showed significantly higher *Oxtr* expression in the raphe of WT animals compared to HET animals (figure 26 e, $p=0.030$). In addition to that, a trend was found in the amygdala for higher *Oxtr* expression in the PS group compared to the control group, independent of *5-Htt* genotype (figure 26 d, $p=0.062$). Results in the other brain regions did not show any significant differences or differences at trend level.

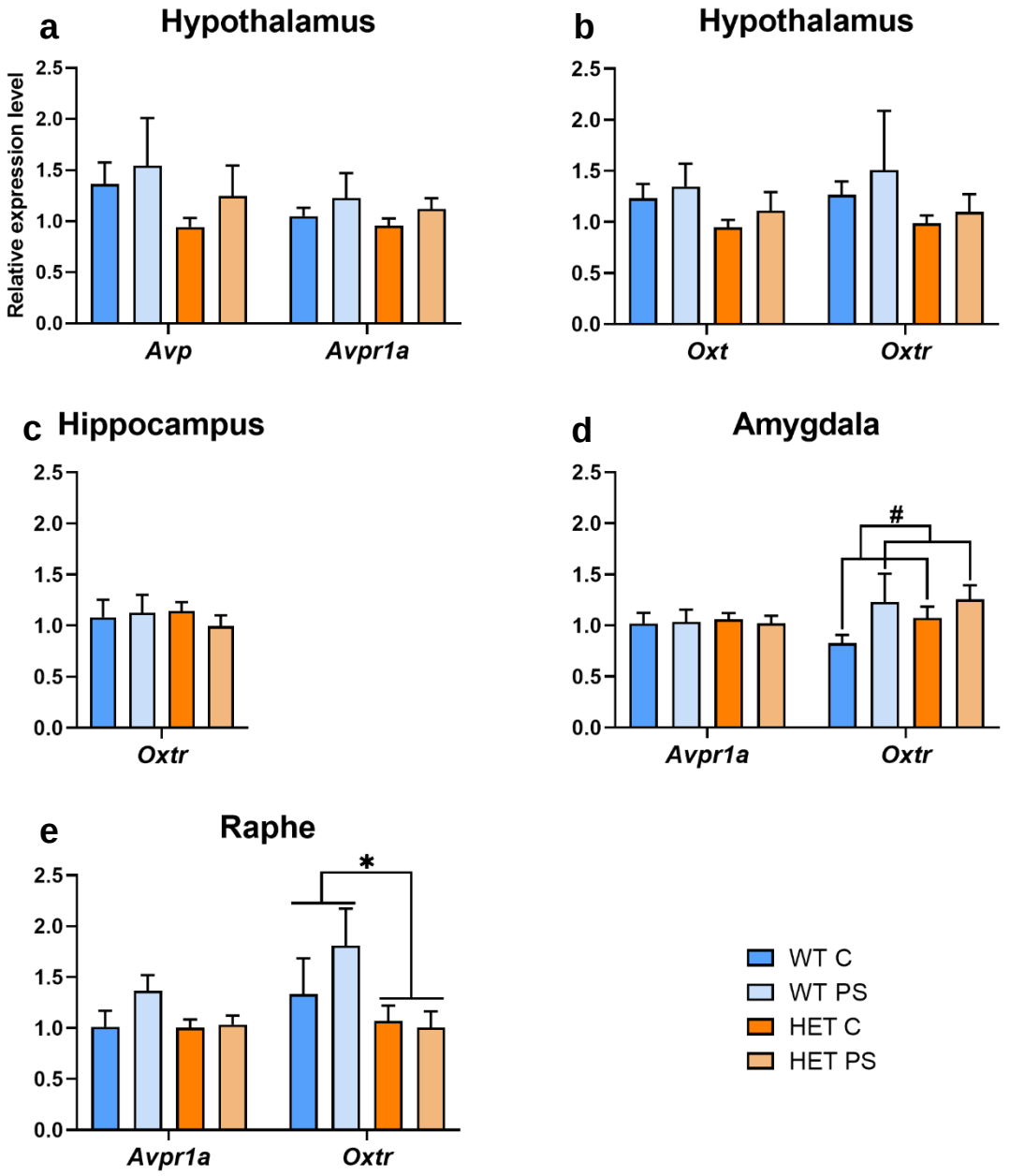

Figure 26: Influence of prenatal stress on *Avp* and *Oxt* gene expression of male mice with both *5-Htt* genotypes.
Relative gene expression of Avpr1a and Oxtr in hypothalamus (a.b), amygdala (c), hippocampus (d) and raphe (e). Relative gene expression of Avp and Oxt in the hypothalamus (a,b). WT wild type animals, HET, 5-HTT heterozygous mice; C control animals without prenatal stress exposure, PS animals with prenatal stress exposure. Statistical analysis was performed using two-way ANOVA, Levene's test and Shapiro-Wilk test (WT Control, n=9; WT PS, n=7; HET Control, n=17; HET PS, n=14). Data are presented as arithmetic means ±SEM. p*<0.05, p#<0.1.

Investigating the serotonergic system (figure 27), more specifically its receptors *Htr1a, Htr2a* and *Htr2c* in all investigated regions and *Tph2* in the raphe region, revealed significantly higher gene expression of *Htr2c* in WT compared to HET animals (figure 27 e, p=0.006). We could not detect any significant differences or differences at trend level in the other investigated regions.

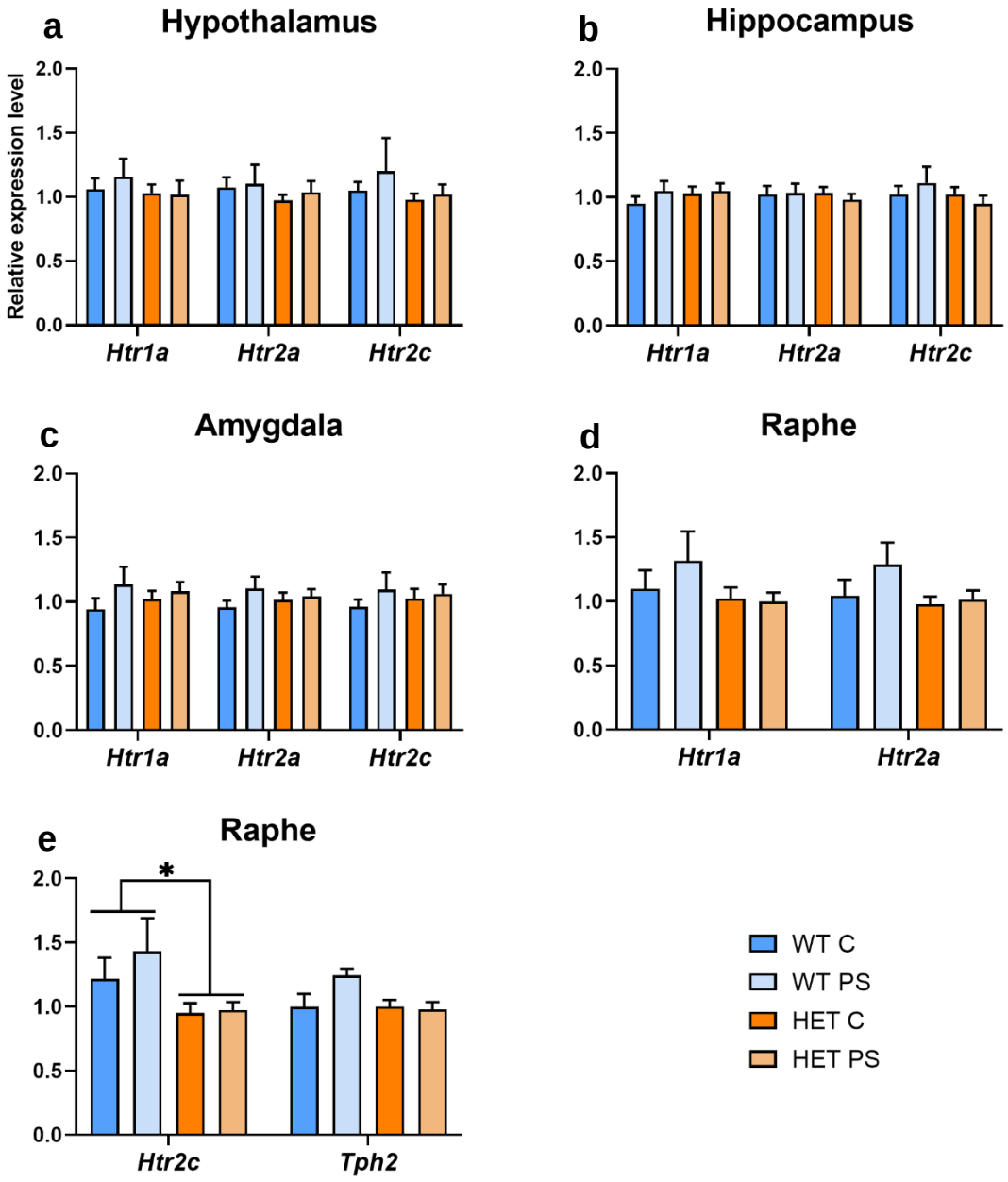

Figure 27: Influence of prenatal stress on the expression of genes belonging to the serotonin system in male mice.
Relative gene expression of Htr1a, Htr2a and Htr2c in hypothalamus (a.), hippocampus (b), amygdala (c) and raphe (d, e). Relative gene expression of Tph2 in the raphe (e). WT wild type animals, HET, 5-HTT heterozygous mice; C control animals without prenatal stress exposure, PS animals with prenatal stress exposure. Statistical analysis was performed using two-way ANOVA, Levene's test and Shapiro-Wilk test (WT Control, n=9; WT PS, n=7; HET Control, n=17; HET PS, n=14) Data are presented as arithmetic means ±SEM.

To compare our results to the male animals of our SFC study, we investigated *Npy a*nd its receptors *Npyr1* and *Npyr2*. Gene expression of *Npyr2* in the amygdala was significantly higher in prenatal stress groups (figure 28 b, p=0.029). In the hippocampus, *Npyr2* gene expression was again higher in PS groups compared to control groups, yielding a trending

result (figure 28 c, p=0.071). The other investigated regions did not show any significant or trending results.

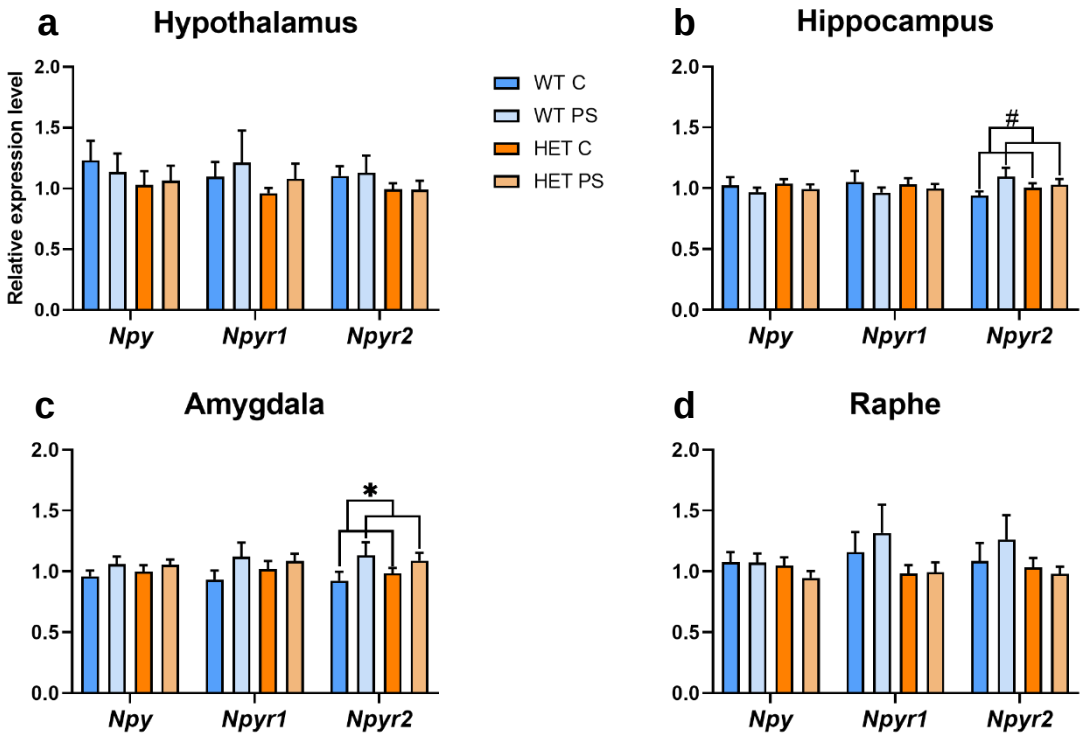

Figure 28: Influence of prenatal stress on gene expression of genes belonging to the Npy system in male mice.
Relative gene expression of Npy, Npyr1 and Npyr2 in hypothalamus (a), amygdala (b), hippocampus (c) and raphe (d). WT wild type animals, HET, 5-HTT heterozygous mice; C control animals without prenatal stress exposure, PS animals with prenatal stress exposure. Statistical analysis was performed using two-way ANOVA, Levene's test and Shapiro-Wilk test (WT Control, n=9; WT PS, n=7; HET Control, n=17; HET PS, n=14) Data are presented as arithmetic means ±SEM. p#<0.1.

All results of the statistical analysis for each group of this analysis are presented in table 17 of the appendix.

3.2.2 Influence of *5-Htt* genotypes on gene expression in naïve male mice

Our analysis of animals that underwent prenatal stress revealed a few interesting gene expression results regarding genotype differences. These could not be attributed to treatment conditions alone, therefore we decided to investigate naïve animals of all three *5-Htt* genotypes, this time also including KO animals which could not be investigated in the PS study due to the breeding scheme only allowing WT and HET offspring. We again investigated hypothalamus, hippocampus, amygdala and raphe, as well as genes belonging to *AVP* and *OXT* systems, the serotonergic system and the NPY system.

Evaluation of mRNA levels of *Avp, Oxt* and their receptors *Avpr1a* and *Oxtr* revealed a significantly higher gene expression of *Avpr1a* in KO animals compared to HET animals, but not to WT animals in the raphe region (figure 29 e, ANOVA p=0.025, Bonferroni Post hoc p=0.021), as well as a trend of higher *Oxtr* gene expression (figure 29 e, ANOVA p=0.063). The other results did not show any significant or trending differences.

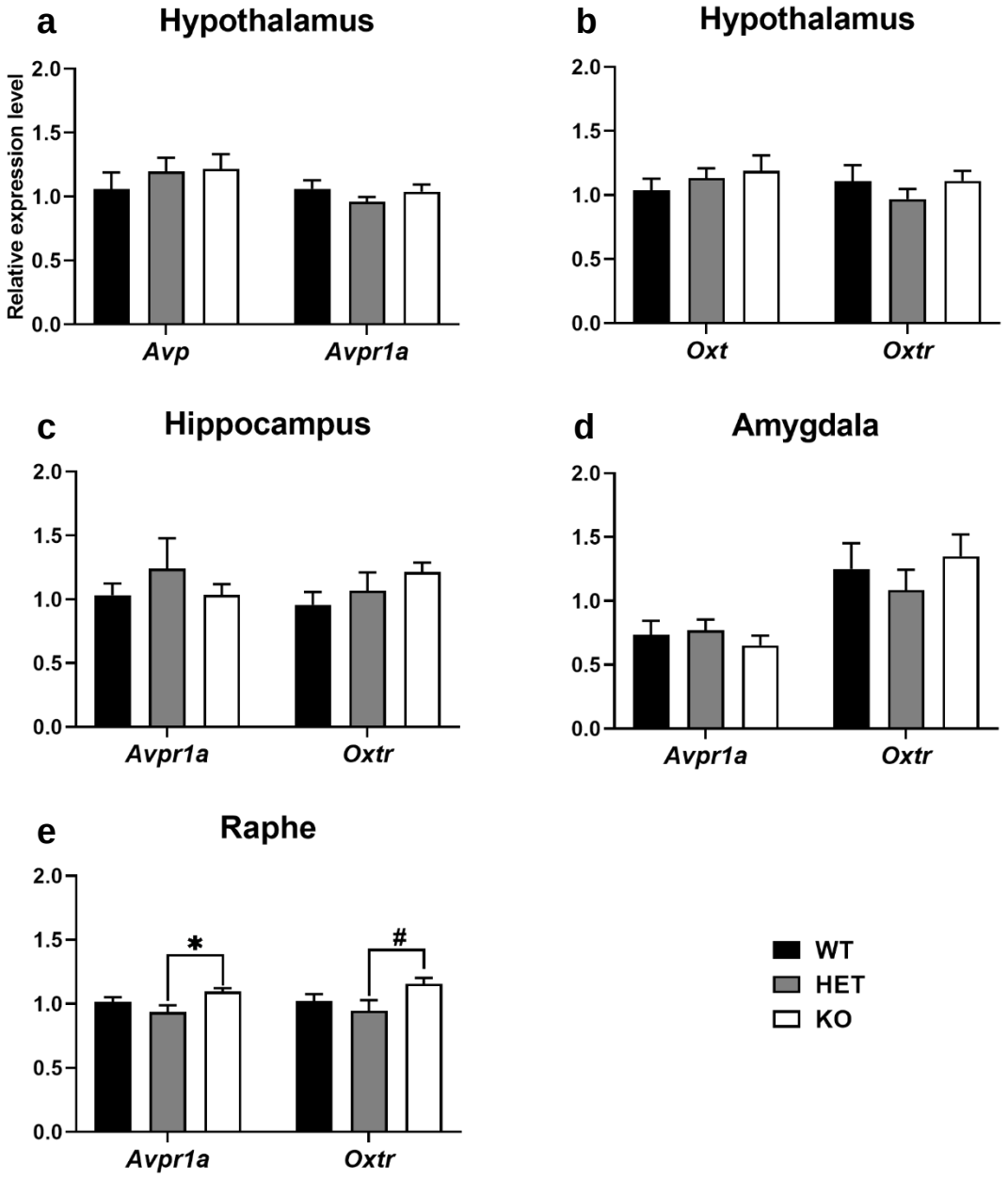

Figure 29: Influence of *5-Htt* genotype on gene expression of AVP and OXT brain systems-related genes of male mice.
Relative gene expression of Avpr1a and Oxtr in hypothalamus (a. b), hippocampus (c), amygdala (d) and raphe (e). Relative gene expression of Avp and Oxt in the hypothalamus (a, b). WT: wild type animals; HET: 5-HTT heterozygous mice, KO: 5-HTT knockout mice. Statistical analysis was performed using one-way ANOVA, Levene's test, Shapiro-Wilk test, and Bonferroni post-hoc test (WT, n=16; HET, n=16; KO, n=15). Data are presented as arithmetic means ±SEM, p*<0.05.

Analysis of the serotonergic system and its receptors *Htr1a, Htr2a* and *Htr2c* did not result in significant or trending differences between the three genotypes (figure 30).

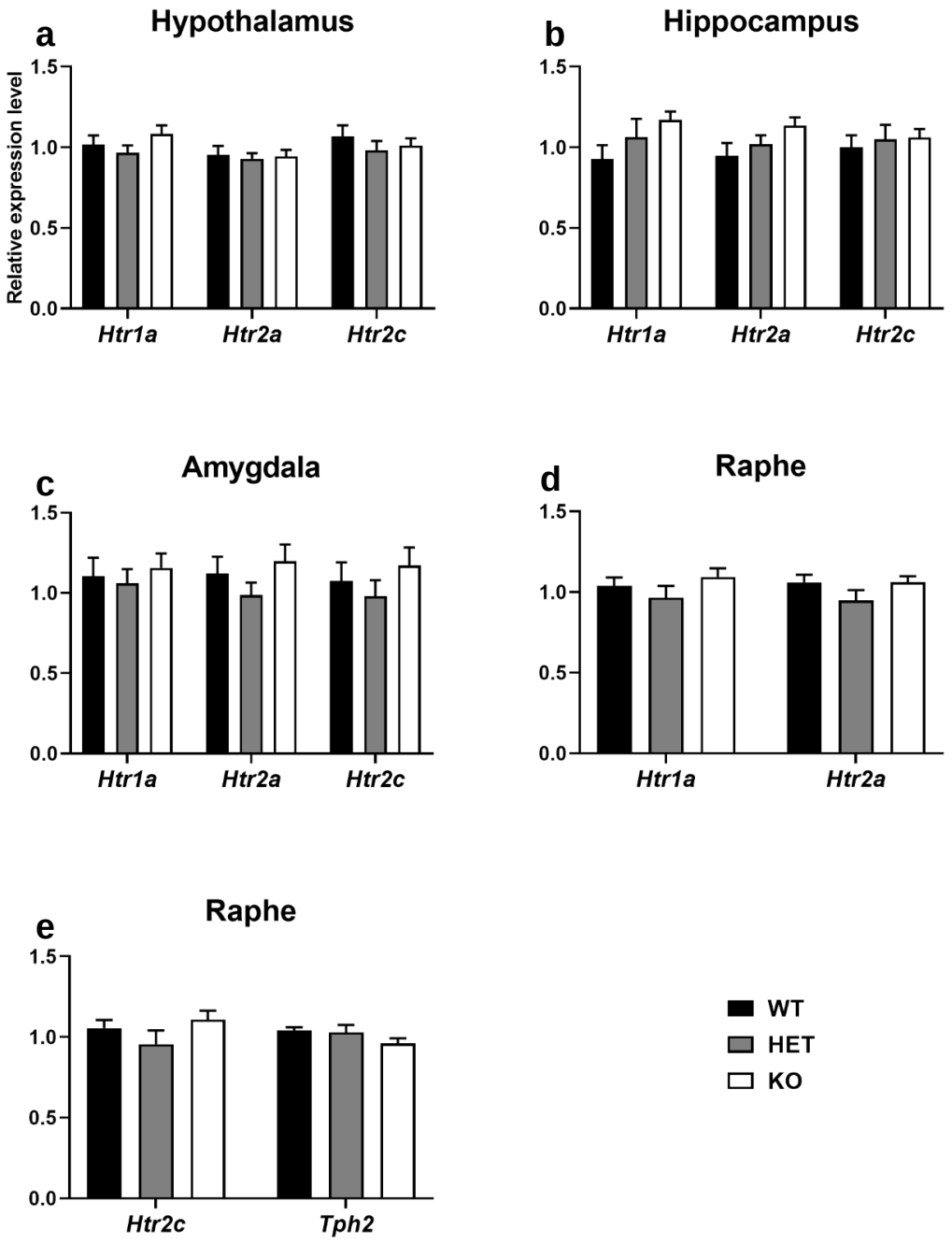

Figure 30: Influence of *5-Htt* genotype on the expression of serotonin system-related genes of naïve male mice.
Relative gene expression of Htr1a, Htr2a and Htr2c in hypothalamus (a), hippocampus (b), amygdala (c) and raphe (d, e). Relative gene expression of Tph2 in the raphe (e). WT: wild type animals; HET: 5-HTT heterozygous mice, KO: 5-HTT knockout mice. Statistical analysis was performed using one-way ANOVA, Levene's test, Shapiro-Wilk test, and Bonferroni post-hoc test (WT, n=16; HET, n=16; KO, n=15). Data are presented as arithmetic means ±SEM.

Results of the *Npy* system and its receptors *Npyr1* and *Npyr2* showed trends for higher gene expression of *Npyr2* in the hippocampus of KO mice compared to WT mice (figure

31 b, p=0.056). Furthermore, the same gene had a trending higher expression in the raphe of KO animals, compared to HET animals (figure 31 d, p=0.068).

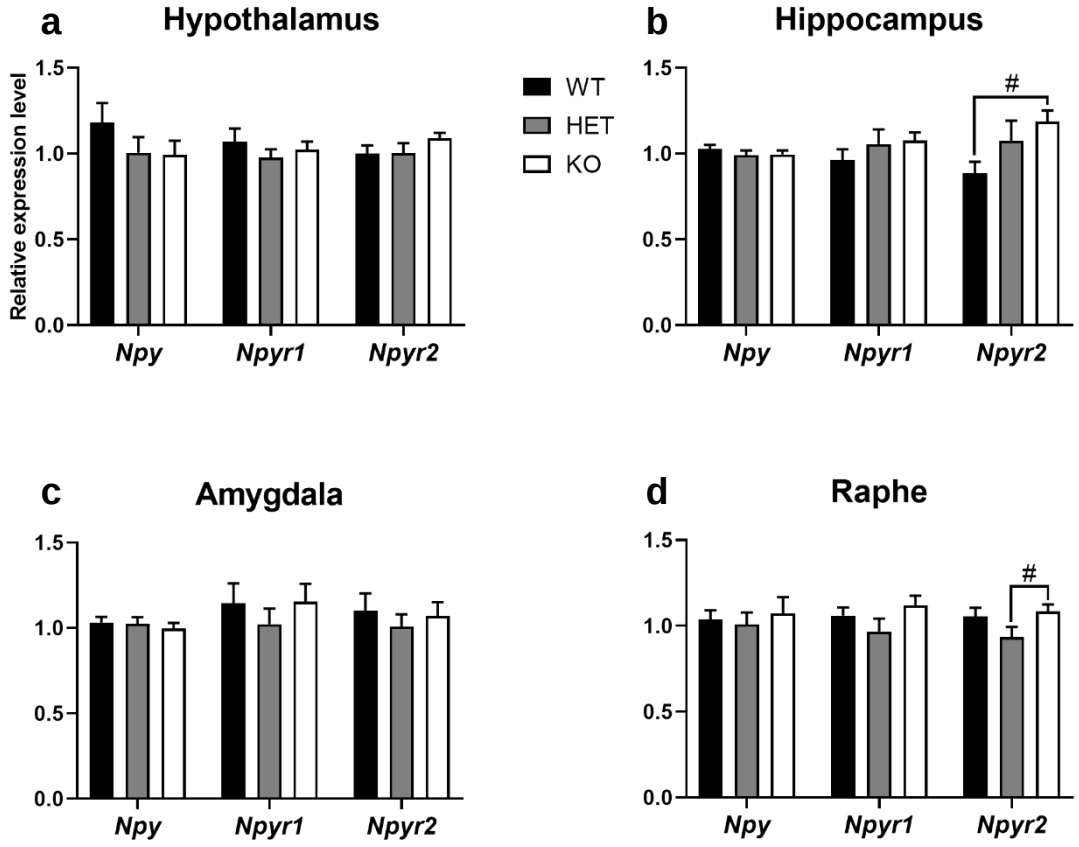

Figure 31: Influence of *5-Htt* genotype on the expression of NPY system-related genes of naïve male mice.
Relative gene expression of Npy, Npyr1 and Npyr2 in hypothalamus (a), hippocampus (b), amygdala (c) and raphe (d). WT: wild type animals; HET: 5-HTT heterozygous mice, KO: 5-HTT knockout mice. Statistical analysis was performed using one-way ANOVA, Levene's test, Shapiro-Wilk test, and Bonferroni post-hoc test (WT, n=16; HET, n=16; KO, n=15). Data are presented as arithmetic means ±SEM.

All results of the statistical analysis for each group of this analysis are presented in table 18 of the appendix.

3.2.3 Influence of estrous cycle stages on the expression of genes related to the social peptides AVP and OXT in naïve female mice of different *5-Htt* genotypes

While investigating the social peptides AVP and OXT in female animals, one should keep in mind that concentrations and gene expression levels of these genes and their receptors can be influenced through estrous cycle stages. Since the original study attributed to the prenatal stress paradigm did not focus on analyzing these systems, estrous cycle stages of the female animals were not determined. It is known that AVP and OXT systems are

influenced by sexual hormones (Lu et al. 2019; Bredewold and Veenema 2018; Albers, Karom, and Whitman 1996). We cannot rule out different cycle stages being responsible for the high *Avp* gene expression in the hippocampus of the HET control group (see fig. 20). While not being able to recreate the prenatal stress paradigm, we were able to provide female animals of all three *5-Htt* genotypes and match them for their estrous cycle stages. While the female estrous cycle of mice can be divided into the four different stages proestrus, estrus, metestrus and diestrus, only the proestrus stage shortly before the ovulation point is associated with high concentrations of the follicle stimulating hormone and estrogen, therefore being referred to as "high". In the other three cycle stages, concentrations of these hormones are lower, which is why they were taken together in the analysis and referred to as "low". We decided to limit this analysis to the AVP and OXT system, to further elucidate our results from the prenatal stress study. Investigated regions were the hypothalamus as source of AVP and OXT production, as well as the hippocampus for AVP expression, where we had detected an unusual gene expression result in our first analysis. Additionally, previous studies found evidence for the occurrence of AVP mRNA in the hippocampus.

Looking at the AVP system, we had to exclude *Avpr1a* in the hippocampus from our analysis, because too many samples had to be excluded. *Avp and Avpr1a* gene expression in the hypothalamus revealed itself to be pretty homogenous between all three investigated genotypes and both low and high estrous cycle groups (figure 32 a & b). The result for *Avp* in the hippocampus however showed big differences between investigated animals, resulting in a very high SEM. This indicates that *Avp* expression is not exclusively influenced by estrous cycle stages. We could not detect any significant or trending differences between the estrous cycle stages or *5-Htt* genotypes.

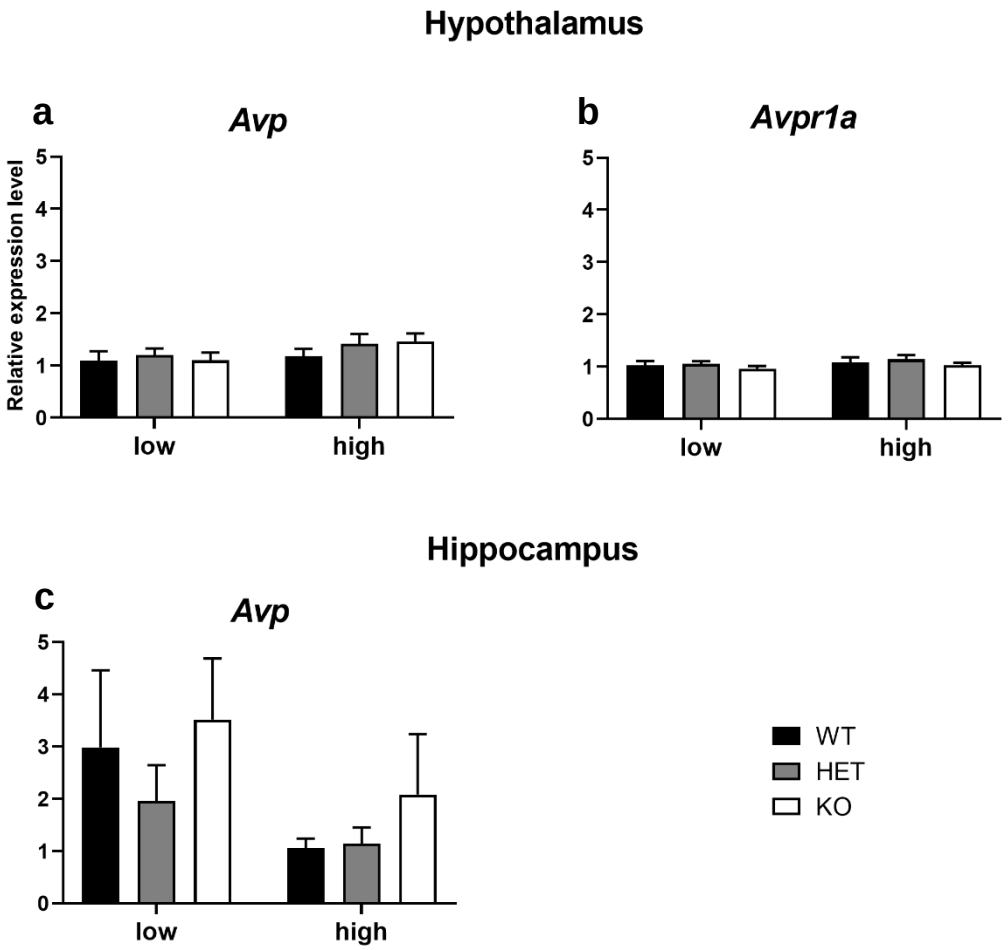

Figure 32: Influence of estrous cycle stages on the expression of genes related to the Avp system in female mice of different *5-Htt* genotypes.
Relative gene expression of Avp in hypothalamus (a) and hippocampus (c). Relative gene expression of Avpr1a in the hypothalamus (b). WT: wild type animals; HET: 5-HTT heterozygous mice, KO: 5-HTT knockout mice. Low = mice with low estrogen levels in estrus, metestrus and diestrus stages of the estrous cycle, high = mice with high estrogen levels in proestrus phase of the estrous cycle. Statistical analysis was performed using one-way ANOVA, Levene's test, Shapiro-Wilk test, and Bonferroni post-hoc test (hippocampus: WT low, n=15; WT high, n=11; HET low, n=14; HET high, n=15; KO low, n=13; KO high, n=12; hypothalamus: WT low, n=15; WT high, n=15; HET low, n=15; HET high, n=16; KO low, n=15; KO high, n=14). Data are presented as arithmetic means ±SEM.

Results for the OXT system revealed a significantly higher gene expression of *Oxt* in the hypothalamus of the high estrous group, compared to the low estrous group (figure 33 a, p=0.022). Contrary to this, we found a lower gene expression of *Oxtr* in the hippocampus of the high estrous group compared to the low estrous group on a highly significant level (figure 33 d, p=0.003). This means that while analyzing gene expression related to the OXT systems in female animals one has to keep in mind that the results might be influenced by the estrous cycle and should ideally adjust the groups according to cycle stages. All results of the statistical analysis for each group of this analysis are presented in table 19 of the appendix.

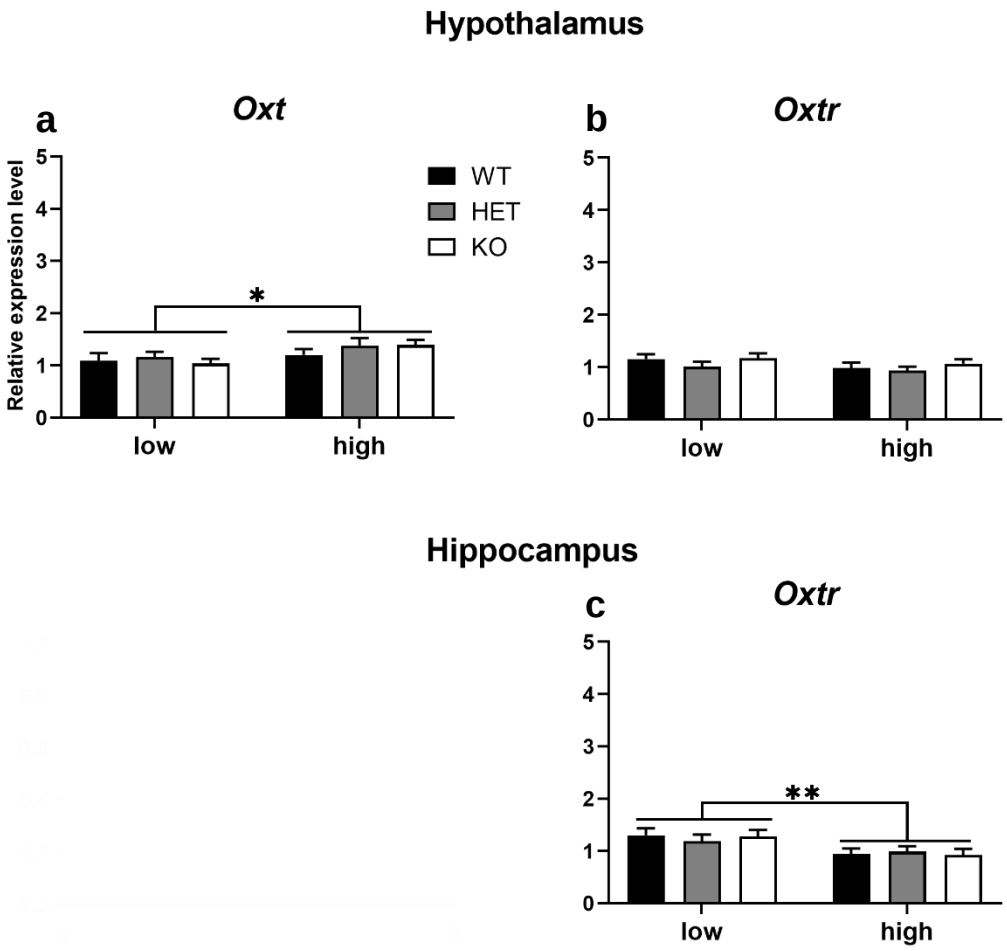

Figure 33: Influence of estrous cycle stages on the expression of genes related to the Oxt system in female mice of different *5-Htt* genotypes.
Relative gene expression of Oxt in hypothalamus (a). Relative gene expression of Oxtr in hypothalamus (b) and hippocampus (c). WT: wild type animals; HET: 5-HTT heterozygous mice, KO: 5-HTT knockout mice. Low = mice with low estrogen levels in estrus, metestrus and diestrus stages of the estrous cycle, high = mice with high estrogen levels in proestrus phase of the estrous cycle. Statistical analysis was performed using two-way ANOVA, Levene's test, Shapiro-Wilk test, and Bonferroni post-hoc test (hippocampus: WT low Oxt, n=15, Oxtr, n=14; WT high Oxt, n=13, Oxtr, n=15; HET low Oxt, n=14, Oxtr, n=15, HET high Oxt, n=15, Oxtr, n=13; KO low, n= 15, KO high, n=14; hypothalamus: WT low, n=15, WT high, n=15; HET low Oxt, n=15, Oxtr n=14; HET high, n=16; KO low n=15; KO high n=14). Data are presented as arithmetic means ±SEM. p*<0.05, p**<0.01.

3.3 Immunofluorescence evaluation of AVP- and OXT-positive cells in naïve male and female mice of all *5-Htt* genotypes

Since little is known about the connection between the serotonergic brain system and the AVP- and OXT- brain systems, we wanted to investigate a possible influence of different *5-Htt* genotypes on the number of AVP- and OXT-positive cells in the PVN. To achieve this, we opted for an immunofluorescence approach by staining the major source of AVP and OXT production, the PVN, in the brains of 5-HTT WT, HET and KO mice of both sexes. Images were taken with a FluoView F1000 Olympus confocal microscope using a 10X UPlanSAPO (NA 0.75 air) objective. Raw images were processed with the internal FluoView imaging software. Counting was performed using the Fiji software. Representative images of a male brain can be seen in figure 34 below.

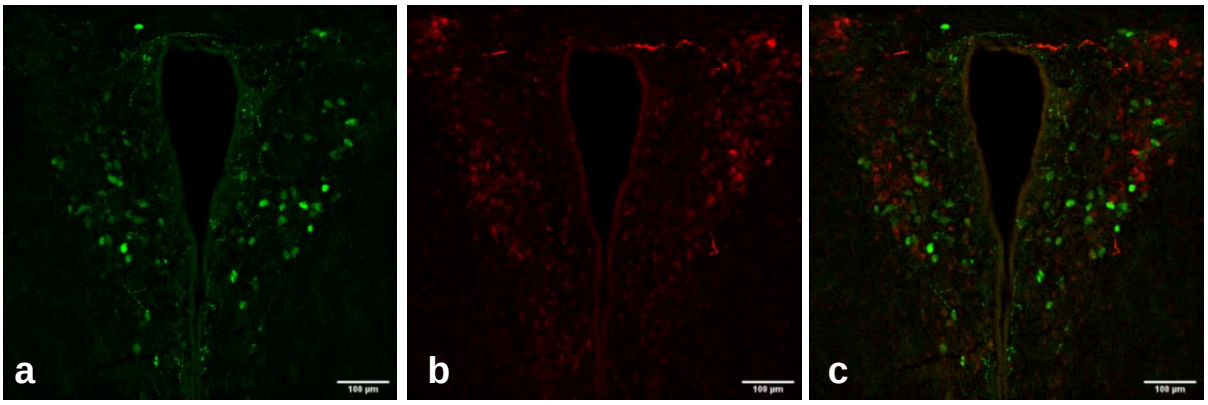

Figure 34: Representative images of an immunofluorescence double staining of AVP and OXT in the PVN.
Immunofluorescence staining of coronal sections with AVP and OXT in a 5-HTT WT male animal. a: oxytocin; b: vasopressin, c: overlay of both images. Scale bars = 100µm.

We analyzed the total number of AVP- and OXT- positive cells by manually counting them and measuring the outline of the PVN next to the outermost positive cells. Cell density was calculated through dividing the number of positive cells by the area size. We wanted to see not only differences between genotypes, but also between the sexes. The results of this analysis are shown in figure 35.

The overall area size of the PVN did not differ recognizably between genotypes or sexes (figure 35 e). Looking at the mean number of cells we found significantly more AVP-positive cells in brains of male mice compared to female mice (figure 35 a, $p=0.039$). Overall cell density, as well as the number of OXT positive cells did not show any significant differences or differences at trend level between groups or genotypes.

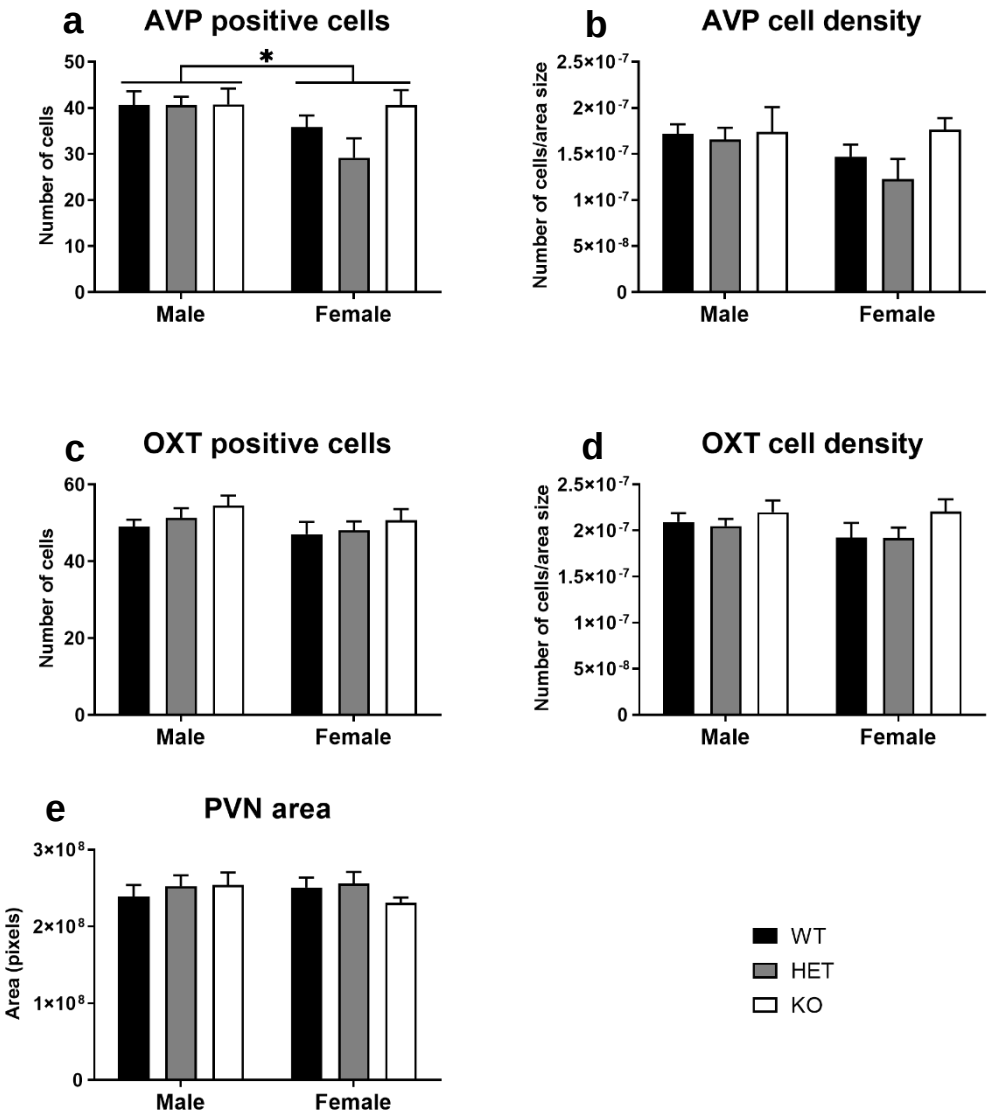

Figure 35: Male animals harbor more AVP-positive cells in the PVN than females.
Mean number of AVP-ir (a) and OXT-ir (c) cells counted in the PVN of 5-HTT WT, HET and KO male and female animals. Mean area of the PVN measured in pixels (e). Mean density of AVP- (b) and OXT-ir cells (d). WT: wild type animals; HET: 5-HTT heterozygous mice, KO: 5-HTT knockout mice. Statistical analysis was performed using two-way ANOVA, Levene's test, Shapiro-Wilk test, and Bonferroni post-hoc test (n=15 for all groups). Data are presented as arithmetic means ±SEM. p*<0.05

4 Discussion

4.1 Social Fear Conditioning influences gene- and protein expression on different levels

The particular influence of SFC on different brain regions being unclear determined us to investigate this question with two different experimental approaches, one looking at mRNA levels and the other one analyzing on the protein level. In this study, we were able to show, that SFC treatment reduced social investigation time almost to zero and specifically induced a fear of social interaction situations as described by (Toth, Neumann, and Slattery 2012a) in their introduction of this animal model. With a focus on different neuromodulatory systems involved in the development of SAD, we tried to get better insights into the processes leading to the disease. Alterations in neuronal activity displayed through differences in gene expression of two different IEGs and cFOS-ir cells strongly suggest an effect of SFC and/or social interaction on these processes.
The additional evaluation of corticosterone concentration in blood plasma did not show a significant elevation for the SFC+ animals compared to SFC- animals. The extraction method used to draw blood from the sacrificed animals could be the reason why the standard deviations were very high. We tried to draw blood directly from the heart, but this was not always possible. Instead, we had to collect trunk blood at the site of decapitation for some animals. It is possible that this led to a distortion of the results obtained.

Our cFOS immunohistochemistry approach visualizes significantly weaker neuronal activation exclusively in the DG of the dorsal hippocampus in SFC+ animals compared to SFC- animals. The human equivalent to the dorsal hippocampus in mice is mostly responsible for cognitive functions like spatial learning (for review see (Zeidman and Maguire 2016)) and the human equivalent to the ventral part of the hippocampus is believed to mediate stress responses (Fanselow and Dong 2010; Bannerman et al. 2004). Changes in protein expression were detectable neither within the other investigated hippocampal areas (CA3 and CA1) nor in the ventral hippocampus. This comes as a surprise since the expression of IEGs has been correlated to enhanced fear memory formation in different settings of fear conditioning (Ressler et al. 2002; Ploski et al. 2010).

Based on the results of altered cFOS protein expression only becoming apparent in the dorsal hippocampus, we investigated only the dorsal part of the hippocampus for a gene expression analysis. Significantly lower IEG expression levels were detected solely in CA3 (*cFos, Fosl2*) and CA1 (*Fosl2*) of the hippocampus, but not in other brain regions, such as the amygdala, septum and PVN. While investigating the NPY system, we could detect the strongest effect of SFC in the septum with significantly increased *Npyr2* mRNA levels in the SFC+ group compared to the SFC- group. Differences on trend level were found for higher *Npyr2* mRNA levels in the BLA as well as higher *Npyr1* mRNA levels in PVN and BLA of SFC+ animals compared to SFC- animals. Contrary to that, a trend was found for

higher *Npy* mRNA levels in the DR of SFC- animals compared to SFC+ animals. No differences in mRNA levels of genes related to the NPY system and the serotonergic system were found in the hippocampus of both SFC- and SFC+ groups.
Not detecting any changes in IEG expression outside of the hippocampus comes as a surprise, since the amygdala is involved in the regulation of fear responses together with the hippocampus and medial prefrontal cortex (mPFC) (Giustino and Maren 2015). In combination with the results on protein level we infer that the dorsal hippocampus serves a stronger function in relation to social interactions, whereas the ventral hippocampus could play a vital role regarding social anxiety and fear.
Both the NPY and the serotonergic brain system play different roles in the regulation of vital systems, like social behavior and fear. Both together regulate food intake and energy homeostasis (Guy, Pelletier, and Bosler 1988). Considering the expression changes of *cFos* and *Fosl2* in the hippocampus it seems that the serotonergic neurotransmission in the hippocampus as well as the NPY system are not involved in the regulation of social fear and/or social interaction. This is in line with previous results showing that social fear expression is not altered by infusion of NPY into the hippocampus (Kornhuber and Zoicas 2021a). However, injecting NPY via intracerebroventricular injection into the brain causes a reduction in the fear induced by SFC through activation of NPY receptors R1 and R2 (Kornhuber and Zoicas 2019). In more detail, NPYR1 serves its effects from the central amygdala, while NPYR2 acts in the DLS (Kornhuber and Zoicas 2021b).
Our result of significantly higher *Npyr2* expression in the septum of SFC+ mice compared to SFC- mice fit the current state of research. The lateral septum is shown to be crucial for the regulation of different areas of social, emotional and fearful behaviors (Sheehan, Chambers, and Russell 2004). Injection of NPY into the DLS led to anxiolytic effects (Kornhuber and Zoicas 2021a), most likely mediated through NPYR1 (Kask, Nguyen, et al. 2001). The expression of social fear, however, seems to be exclusively mediated through NPYR2 (Kornhuber and Zoicas 2021b). Apart from these studies the literature remains contradictory regarding the exact function of NPYR2, especially regarding different brain areas. Interestingly, initial studies using Npyr2 KO mice showed decreased levels of anxiety-like behavior (Redrobe et al. 2003; Tschenett et al. 2003). However, another study with Npyr2 KO mice on a different genetic background was unable to replicate these results (Zambello et al. 2011). The results we obtained in this study hint towards an involvement of NPYR2 in the regulation of responses to social fear through the septum region.

The different mRNA activation time points of *cFos* and *Fosl2*, as well as a generally time-shifted activation of mRNA and protein are limitations for a clear interpretation of our results. We sacrificed the animals two hours after social fear assessment, which is an ideal point to investigate cFOS protein activation, that reaches its peak 90-120 min after a stimulus (Kovács 1998). The cFos mRNA level however, reaches its highest point 30 min after neuronal activation (Kovács 2008), while Fosl2 mRNA expression is at its highest point between one and two hours after neuronal activation (Honkaniemi et al. 1994).

The contrast between our mRNA results and the number of cFos-ir cells could also be rooted in different methodological approaches. We were able to distinguish singular cells in our IHC approach and only took cells into account, which were strongly stained and therefore clearly cFos-positive. On the other hand, the extraction of whole regions with laser microdissection leads to a more heterogeneous cell population regarding cFos expression levels. In general, interpretation of the IEG results is very difficult. Due to a lack of a control group without any kind of social interaction with conspecifics, it is not possible to interpret the direction of changes in gene expression levels. An additional study would be necessary to determine if the expression of IEGs is upregulated after social interaction or downregulated after social fear conditioning experience.

4.2 Prenatal stress affects gene expression of 5-HTT deficient mice

Female mice. It is known that prenatal stress can have negative consequences on the unborn fetus and increase the risk of psychiatric disorders in later life. Furthermore, specific variants in a polymorphism of the human *5-HTT* gene increase the risk for anxiety disorders. However, the links between prenatal stress exposure and gene expression of different neurotransmitter systems are not well understood. In her PhD-thesis, (Schraut 2015) applied a model of prenatal restraint stress combined with exposure to bright light and water. The female offspring was subjected to different behavioral tests. According to their behavior in the three-chamber sociability test, prenatally stressed animals were categorized in "vulnerable" and "resilient" groups in response to the stress experience. Animals vulnerable to the stress paradigm showed noticeably less social interaction and were therefore denominated "unsocial". Animals resilient to the stress experience were called "social". Based on this initial study, we used brain tissue of the animals included in this study, to perform a gene expression analysis. We were able to see a trend for the upregulation of *Avpr1a* in the raphe region of prenatally stressed mice that displayed behavioral abnormalities consistent with "unsocial" behavior. This is in line with the theory of AVP regulating passive stress coping mechanisms (Neumann and Landgraf 2012).

Not taking the "social" and "unsocial" behavioral groups into account and limiting the analysis to prenatal stress- and genotype effects, we could show that prenatal stress led to a significant upregulation of *Oxtr* in the hippocampus. Additionally, the expression of *Tph2*, was significantly lower in female mice heterozygous for the *5Htt* knockout (HET mice), compared to the WT mice. The expression of *Avp* in the hippocampus delivered quite a puzzling result. The HET control group showed higher gene expression of *Avp* than all other investigated groups unrelated to treatment or any other possibly influencing factors.

The upregulation of *Oxtr* in the hippocampus of prenatally stressed mice could hint towards an influence of the OXTR in memory consolidation, given the role that the hippocampus plays in response to aversive events. Since this first study was only performed in female offspring of the prenatal stress paradigm, we wanted to further

investigate, whether the change in *Oxtr* expression could also be found in the brains of male mice exposed to prenatal stress. In addition to that, other gene expression changes are possible.
TPH2 is the rate-limiting enzyme of the serotonin synthesis. Our finding of higher *Tph2* expression in HET mice was not in line with the results of a study that also investigated Tph2 in mice of different *5Htt* genotypes. They reported similar expression levels in 5HTT WT and KO mice (Kim et al. 2005). However, they chose a different approach and detected protein levels via Western Blot. The effect we found was independent from the study design. This allowed us to investigate *Tph2* expression in the male mice from the PS study, as well as naïve male animals with all three *5Htt* genotypes as a next step. Results of the following study with naïve male mice are discussed in chapter 4.3.
The evaluation of *Avp* expression in hippocampal regions has to be taken with caution, since to date only a few sources indicate the presence of *Avp* mRNA in the hippocampus. However, influence of the estrous cycle of both AVP and OXT systems is undisputed. We presumed, that different estrous cycle stages of the mice assigned to this group could have affected and distorted the result. A detailed discussion of this result and the following study is presented in chapter 4.4.
Surprisingly, even though the hypothalamus region is heavily involved in the regulation of processes related to stress, no alterations in the expression of above-mentioned genes could be found in this region. This could also be due to differences in estrous cycle stages, which were not assessed in the original study. In addition to that no 5-HTT KO animals, which are more susceptible to effects of stress, were investigated.

Male mice. In a second step, we repeated our investigation with the brains of the male offspring, which had not been subjected to behavioral testing, but were exposed to the prenatal stress paradigm. Since we are trying to identify possible links between the AVP/OXT brain system and the serotonergic system, this could be a first hint towards a relationship between them.
We found a trend of higher *Oxtr* expression in the amygdala of PS mice, compared to the control mice. Our analysis of the NPY system-related genes revealed a trend of higher *Npyr2* expression in the hippocampus of both PS groups compared to the control groups, as well as a significantly higher expression of *Npyr2* in the amygdala of PS mice compared to control mice. Gene expression of the serotonin receptors *Htr1a*, *2a*, as well as *Tph2* was not altered in any of the investigated regions. However, we were able to detect a significantly higher gene expression of *Htr2c* in the raphe of WT animals, compared to HET animals.
Interestingly, regarding the regulatory function of AVP and OXT in social behavior, the alteration of higher *Oxtr* expression in the amygdala seems to be opposite of the result we obtained in female mice, where *Avpr1a* expression was upregulated on trend level in the amygdala of PS mice. A trending result is not strong enough to propose a sex difference in hormonal regulation after stressful exposure, but specific investigations in this direction could highlight the distinctions between male and female stress response.

The alterations found for the expression of *Npyr2* in PS mice corroborate our results of the SFC study, where we found a trend of higher *Npyr2* expression in the amygdala, as well as a significantly higher expression in the septum of SFC+ animals. This further emphasizes the role of *Npyr2* in the regulation of stressful experiences and its importance for anxiety-related behavior. Because the prenatal stress exposure dates back a much longer timeframe than the SFC exposure, this process might be long term. A limiting factor of the study is the fact that the NPY system was only investigated in male mice. Possible sex differences could therefore not be investigated.
We were not able to replicate the genotype difference of *Tph2* expression in the raphe of female mice. Our finding of higher expression in the WT group of female mice compared to HET mice could either have been a random result with no further importance, or the behavioral tests employed on the female mice set this process into motion. Since the male mice were not subjected to behavioral testing, this could explain why we were not able to see the same effect regarding *Tph2* expression. Our finding of significantly higher gene expression of *Htr2c* in the raphe of WT animals, compared to HET animals could be an indirect consequence of the 5-HTT deficiency, but cannot be explained further at the moment.

4.3 Different *5-Htt* genotypes influence gene expression of social peptides in naïve male mice

Our initial goal to search for connections between the serotonergic system and the AVP/OXT system in combination with the prenatal stress study delivering some results regarding genotype differences led us to another follow-up study. We aimed to investigate naïve male mice of all three *5-Htt* genotypes and to analyze gene expression levels of the already established genes, detached from the influence of negative life events. The raphe showed significantly higher *Avpr1a* expression in KO animals compared to HET animals, but not to WT animals. The same effect was found for *Oxtr* expression differences on trend level. We also found two trends for the gene expression of the *Npyr2* receptor in both the hippocampus and the raphe region. KO animals had higher gene expression compared to WT animals in the hippocampus and compared to HET animals in the raphe.
Gene expression changes of *Avpr1a* and *Oxtr* in the raphe are insofar interesting, as the raphe region is the source of serotonin production, which is heavily affected through the 5-HTT knockout. However, we cannot explain yet why significant, as well as differences on trend level only become apparent in comparison to the HET group, but not to WT animals. Considering the lack of this effect in comparison to WT animals leaves the possibility that this is result is an outlier.
Even though the expression changes for *Npyr2* are too weak to draw any firm conclusions from them, it seems like the *5-Htt* knockout affects this neurotransmitter system. Interestingly, again the *Npyr2* receptor is affected, which was also influenced by SFC and altered in animals that were exposed to stressful experience.
The occurrence of only weak alterations on gene expression level suggests that the AVP/OXT brain system is not directly influenced through *5-Htt* genotype. However, the fact that some of the investigated genes showed altered gene expression influenced by

alterations in the serotonergic system does not rule out the possibility of connections between the neurotransmitter systems. Further investigation is required to determine a potential occurrence and, if so, the possible nature of this connection.

4.4 Influence of estrous cycle stages on AVP and OXT system gene expression

In our first gene expression study with female prenatally stressed animals we found higher expression of *Avp* in the hippocampus region of one control group compared to all other groups. Since we did not find any changes in gene expression in any other brain region, related to the prenatal stress paradigm or *5-Htt* genotype, we presumed an involvement of other factors influencing the fluctuation of *Avp* gene expression. Since it is known that both AVP and OXT are interrelated with the estrous cycle, we wanted to investigate female mice of all *5-Htt* genotypes in both low and high estrous cycle stages, to determine the possible influence of estrous cycle on *Avp* and *Oxt* gene expression.

The estrous cycle of mice is comprised of four different stages, proestrus, estrous, metestrus and diestrus (Allen 1922). The stages can be differentiated through cytological staining and are characterized through presence and character of different cell types (McLean et al. 2012; Cora, Kooistra, and Travlos 2015). Highest concentration of the ovarian hormones estrogen and progesterone is found during the proestrous phase (Smith, Freeman, and Neill 1975; Butcher, Collins, and Fugo 1974). Therefore, we divided the analyzed animals in a ”high” estrogen group, containing animals in the proestrus phase and a “low” estrogen group, containing animals in all other phases of the estrous cycle.

Our analysis revealed significantly higher gene expression of *Oxt* in the hypothalamus of the high estrous group, as well as significantly higher gene expression of *Oxtr* in the hippocampus of the low estrous group, whereas no significant or trending differences wee found for the AVP system.

The interrelation between AVP and OXT brain systems and the estrous cycle is well studied. In rats, expression of AVP in lateral septum and lateral habenular nucleus is highly depending on the circulation of gonadal steroids (de Vries, Buijs, and Sluiter 1984). In female rats, administration of oxytocin during specific estrous cycle stages can influence behavior and improve estrous cycle dependent reduction of conditioned safety memory (Kreutzmann and Fendt 2021). OXTR binding in the ventromedial nucleus of the hypothalamus (VMN) of female rats is influenced by sexual hormones (Johnson 1992). In rats, the promotor region of the *Oxt* gene harbors a response element to estrogen (Peter et al. 1990; Adan et al. 1993; Quiñones-Jenab et al. 1997), which suggests possible interaction points between the oxytocin system and sexual hormones (Liu et al. 2020). Repeated exposure to sexual hormones estradiol and progesterone for two weeks, followed by withdrawal of progesterone, led to an increase in oxytocin mRNA in the hypothalamus in female and castrated male rats (Amico et al. 1995; Thomas and Amico 1996; Amico, Thomas, and Hollingshead 1997). Binding and expression of the OXTR changes dynamically throughout the estrous cycle phases and is dependent on sex steroids (Dumais et al. 2013; Jurek and Neumann 2018). The number of oxytocin receptor

binding sites in the uterus of rats differs between estrous cycle phases (Larcher et al. 1995).
Taken together with our previous results, which showed higher gene expression of *Avp* in the hippocampus of a control group with no treatment conditions compared to the other treatment groups, this supports our theory of different estrous cycle stages influencing this result. Even though we were not able to replicate our previous results, the presence of significant differences in gene expression in naïve animals is a strong indicator, that the estrous cycle might have been the reason we obtained this otherwise unexplainable result.

4.5 AVP- and OXT-positive cells in mice of all *5-Htt* genotypes

Next to our mostly mRNA-based gene expression approaches, we wanted to investigate the protein level and search for connections between AVP and OXT brain systems with the serotonergic system. We therefore opted for an immunofluorescence approach to analyze AVP- and OXT-ir cells in the PVN. We utilized our animal model of 5-HTT KO mice with all three genotypes (WT, HET, KO) to evaluate whether reduced levels of 5-HTT or its complete absence would influence the number of AVP and OXT positive cells in the PVN. The evaluation revealed significantly more AVP-positive cells in male animals compared to female animals, independent from the *5-Htt* genotype. The number of OXT-ir cells showed no significant differences or differences on trend level, neither between the sexes nor between *5-Htt* genotypes.
Sex differences regarding AVP and OXT protein levels have already been found in different species. In rats, male animals were found to have significantly more AVP-ir cells in the lateral septum and lateral habenular nucleus, compared to females (De Vries and Panzica 2006). Immunohistochemical studies in mandarin voles showed significantly more AVP-ir neurons in the PVN of dominant male voles compared to females (Qiao et al. 2014). A study with a small sample of human brains also found larger AVP neurons in the brains of younger male individuals compared to female individuals (Ishunina and Swaab 1999). Taken together, the available studies support our results of a higher number of AVP-positive cells in male animals, as similar results have been found across different rodent species.
Even though we could not see any differences related to the *5-Htt* genotype, looking at AVP and OXT connections in the brain reveals a few locations that could serve as contact points with the serotonergic system. It is known that projections of AVP arising from neurons in the extended amygdala, innervate the DR (Rood and De Vries 2011), where most of the serotonin neurons projecting to the forebrain are seated (Azmitia and Segal 1978). Excitation of serotonergic neurons in the DR is modulated indirectly through AVPR1A (Rood and Beck 2014). In humans, administration of OXT modulates the 5-HT system, by increasing the non-displaceable binding potential of a 5-Htr1a antagonist, therefore interfering with 5-HT neurotransmission (Mottolese et al. 2014). Infusion of OXT facilitates the release of serotonin in the median raphe and reduces anxiety-related behavior, likely through activation of OXTR in serotonergic neurons (Yoshida et al. 2009). Combined activation of oxytocin and 5-HT neurotransmission in the nucleus accumbens

regulate social reward processes after social interaction via 5-HTR1B receptors (Dölen et al. 2013). On the other hand, serotonin is able to stimulate the release of AVP and OXT to peripheral blood (Iovino and Steardo 1985; Anderson, Martin, and Ramage 1992). In humans, the use of 5-HT reuptake inhibitors was shown to reduce AVP levels in cerebrospinal fluid (CSF) (De Bellis et al. 1993; Altemus et al. 1994).

5 Outlook

The results of our fear conditioning study revealed a possible involvement of the dorsal hippocampus in the expression of social fear. This process is unlikely regulated through the hippocampus alone, due to the fact that general fear response is achieved through a combination of hippocampus, amygdala and mPFC activation (Giustino and Maren 2015; Tanimizu et al. 2017). Since the analysis of other investigated brain regions was limited to gene expression levels, further investigation of cFOS immunoreactivity in brain regions of fear regulation like BLA, central amygdala, infralimbic and prelimbic cortices will highlight which of these regions are involved. A question that still has to be determined is the interpretation whether cFOS immunoreactivity and gene expression in hippocampal regions was either elevated in SFC- animals or lowered in SFC+ animals. While current literature is pointing towards lowered neuronal activity and gene expression in SFC+ animals, due to the fact that social interaction alone was not found to alter cFOS immunoreactivity in the hippocampus (Tanimizu et al. 2017; Lüscher Dias et al. 2016), it is necessary to repeat the experiment with a third group without any social contact, in order to determine basal cFOS immunoreaction and gene expression levels. In addition to that, the time point of sacrifice after social fear assessment should be accommodated to investigate either mRNA or protein activation of the investigated IEGs, since a time-shift can be observed. Furthermore, our initial study was limited to male mice. However, the prevalence of SAD is much higher in women compared to men, which would make a repetition of this study in female mice worthwhile.

The second experimental approach involving prenatal stress in a mouse model of 5-HTT deficiency affected AVP and OXT brain systems only in female, but not in male animals. Since anxiety disorders are more prevalent in women and the influence of prenatal stress on the risk of developing psychiatric disorders in general and anxiety disorders in particular is well-known, further studies investigating the influence of prenatal stress on AVP and OXT brain systems could be very promising. Future studies could include additional brain regions like the septum, which is involved in regulating the response to social fear, as well as controlling mice for the estrous cycle, which can be very influential to *Avp* and *Oxt* expression, as is described below. The *5-Htt* genotype alone did not show an influence on AVP and OXT system gene expression, neither in male nor in female mice, and it is known that prenatal stress alone has an impact on AVP and OXT, at least in rats (de Souza et al. 2013). It would be interesting to see whether prenatal stress alone induces the same alterations, if the *5-Htt* deficiency plays a role in this as well and, if so, to which extent. A coincidental finding of *Npyr2* expression being elevated in the amygdala of prenatally stressed males connects this study to the SFC study, where the expression was elevated in the septum. In the past, NPYR2 has been implemented in the regulation of the response to social fear and could also be involved in the regulation of effects caused by prenatal stress. Studies investigating NPYR2 as a key regulator of stress responses could shine a closer light on the function of this receptor. LMD as a tool which is able to investigate very distinct brain areas and even single cells could help to identify and characterize NPYR2-positive neurons and in the end possible networks connected to

them. In general, LMD has proven to be a reliable tool to analyze small brain regions and even subsets of regions, which is a strong method to unveil connections only present in small neuronal populations.

The observed changes of gene expression in AVP and OXT brain systems related to estrous cycle stages, as well as general sex differences between the number of AVP- and OXT-positive neurons in the PVN are very important to take into consideration for further research regarding these peptide hormones. It is very important to always check and control for the estrous cycle stages while investigating female animals, to avoid a distorted picture and possible wrong interpretation of achieved results. In human research, the estrous cycle is already taken into consideration in a lot of studies regarding brain research. At least for AVP and OXT, this should be implemented in animal research as well. A big limitation of the prenatal gene expression study with female mice is the fact that we were not able to control for the estrous cycle.
Further research is needed into the background of the mechanisms behind the sex difference for AVP and OXT functions in different brain areas. Research is mostly focused on ratio and differences of AVP and OXT, but the mechanisms behind the sex differences remain unclear. Our result of a higher number of AVP-ir cells in the PVN of males compared to females has not been shown in mice before.

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6 Appendix

6.1 Tables of the appendix

Table 14: Relative expression data and statistical analysis of qPCR data from the granule cell layer of the DG, the pyramidal cell layer of CA3 and CA1, the septum, the PVN, the BLA and the DR. Relative expression is presented as arithmetic means ± SEM. Bold entries highlight a significant p-value. Entries in italics indicate the trend of a significant difference. SFC-, unconditioned control mice; SFC+, conditioned socially fearful mice.

Septum							
Gene	Mean (±SEM) of different treatments		Two-sample t-Test *p*-value	Mann-Whitney-U Test *p*-value	Levene's-Test *p-value*	Shapiro-Wilk Test *p-value*	
	SFC- (n=10)	SFC+ (n=10)	SFC+ vs. SFC-	SFC+ vs. SFC-		SFC-	SFC+
Arc	0.99 (±0.07)	0.91 (±0.05)	0.329		0.414	0.774	0.605
Avpr1a	0.91 (±0.07)	1.12 (±0.12)	0.163		0.106	0.379	0.503
cFos	0.82 (±0.04)	0.82 (±0.04)	0.962		0.812	0.827	0.355
Fosl2	1.19 (±0.11)	1.14 (±0.07)		0.853	0.200	0.491	**0.017**
Htr1a	0.98 (±0.03)	1.06 (±0.05)	0.219		0.281	0.873	0.059
Htr2a	1.09 (±0.03)	1.08 (±0.05)	0.882		0.312	0.973	0.934
Htr2c	0.96 (±0.02)	1.04 (±0.04)	*0.079*		*0.097*	*0.069*	0.709
Npy	1.18 (±0.11)	0.94 (±0.11)		0.130	0.578	**0.048**	0.065
Npyr1	1.08 (±0.07)	1.04 (±0.07)	0.702		0.613	0.692	0.147
Npyr2	0.93 (±0.06)	1.21 (±0.10)		**0.035**	0.222	**0.015**	0.915
Oxtr	1.01 (±0.04)	1.00 (±0.04)	0.901		0.525	0.348	0.176
Hypothalamus - paraventricular nucleus							
Gene	Mean (±SEM) of different treatments		Two-sample t-Test *p*-value	Mann-Whitney-U Test *p*-value	Levene's-Test *p-value*	Shapiro-Wilk Test *p-value*	
	SFC- (n=10)	SFC+ (n=10)	SFC+ vs. SFC-	SFC+ vs. SFC-		SFC-	SFC+
Arc	1.07 (±0.14)	1.06 (±0.06)		0.436	0.146	1.07 (±0.14)	1.06 (±0.06)
Avp	1.09 (±0.17)	0.91 (±0.16)	0.456		0.769	1.09 (±0.17)	0.91 (±0.16)
Avpr1a	1.01 (±0.08)	1.06 (±0.11)	0.737		0.161	1.01 (±0.08)	1.06 (±0.11)

cFos	0.99 (±0.12)	1.09 (±0.07)	0.463		*0.054*	0.99 (±0.12)	1.09 (±0.07)
Fosl2	0.98 (±0.05)	1.05 (±0.11)		0.631	**0.017**	0.98 (±0.05)	1.05 (±0.11)
Htr1a	0.901 (±0.07)	1.09 (±0.09)		*0.089*	0.765	0.901 (±0.07)	1.09 (±0.09)
Htr2a	0.97 (±0.05)	1.16 (±0.11)		**0.023**	0.246	0.97 (±0.05)	1.16 (±0.11)
Htr2c	0.97 (±0.05)	1.02 (±0.04)		0.353	0.858	0.97 (±0.05)	1.02 (±0.04)
Npy	1.07 (±0.10)	0.93 (±0.09)	0.328		0.768	1.07 (±0.10)	0.93 (±0.09)
Npyr1	0.93 (±0.05)	1.13 (±0.09)	*0.064*		0.370	0.93 (±0.05)	1.13 (±0.09)
Npyr2	0.94 (±0.07)	1.04 (±0.11)	0.436		0.385	0.94 (±0.07)	1.04 (±0.11)
Oxt	0.97 (±0.15)	0.99 (±0.14)	0.922		0.558	0.97 (±0.15)	0.99 (±0.14)
Oxtr	1.00 (±0.07)	1.16 (±0.08)	0.188		0.958	1.00 (±0.07)	1.16 (±0.08)
Hippocampus - DG							
Gene	Mean (±SEM) of different treatments		Two-sample t-test *p*-value	Mann-Whitney-U Test *p*-value	Levene's-Test *p-value*	Shapiro-Wilk Test *p-value*	
	SFC- (n=10)	SFC+ (n=10)	SFC+ vs. SFC-	SFC+ vs. SFC-		SFC-	SFC+
Arc	1.15 (±0.13)	1.06 (±0.21)	0.718		0.106	0.142	0.201
Avpr1a	0.95 (±0.07)	0.89 (±0.21)	0.646		0.506	0.341	0.490
cFos	1.2 (±0.18)	1.11 (±0.17)	0.715		0.731	0.142	0.131
Fosl2	1.3 (±0.17)	1.06 (±0.20)	0.382		0.600	0.056	0.120
Htr1a	1.07 (±0.07)	0.97 (±0.14)	0.516		0.120	0.350	0.239
Htr2a	1.02 (±0.04)	1.06 (±0.08)	0.682		**0.026**	0.619	0.345
Npy	1.07 (±0.05)	1.05 (±0.12)	0.241		0.63	0.758	0.696
Npyr1	1.08 (±0.05)	1.0 (±0.06)	0.374		0.523	0.380	0.855
Npyr2	1.03 (±0.04)	1.0 (±0.09)		0.364	**0.021**	**0.02**	0.499
Oxtr	1.09 (±0.13)	1.07 (±0.13)	0.897		0.797	0.357	0.237
Hippocampus – CA3							
Gene	Mean (±SEM) of different treatments		Two-sample t-test *p*-value	Mann-Whitney-U Test *p*-value	Levene's-Test *p-value*	Shapiro-Wilk Test *p-value*	
	SFC- (n=10)	SFC+ (n=10)	SFC+ vs. SFC-	SFC+ vs. SFC-		SFC-	SFC+
Arc	0.82 (±0.05)	0.95 (±0.07)	0.143		0.366	0.330	0.245

cFos	0.83 (±0.05)	1.14 (±0.08)	**0.006**		*0.068*	0.768	0.557
Fosl2	0.96 (±0.08)	1.24 (±0.10)	**0.037**		0.485	0.551	0.908
Htr1a	1.09 (±0.07)	1.05 (±0.07)	0.591		0.308	0.779	0.883
Htr2a	1.0 (±0.03)	1.04 (±0.04)	0.472		*0.096*	0.376	0.667
Npy	1.0 (±0.04)	1.02 (±0.06)	0.799		*0.092*	0.204	0.653
Npyr1	0.97 (±0.03)	1.0 (±0.06)	0.712		0.204	0.285	0.487
Npyr2	1.01 (±0.03)	1.02 (±0.03)	0.881		0.367	0.412	0.133
Oxtr	1.07 (±0.03)	1.04 (±0.07)		0.631	0.045	0.638	0.461
Hippocampus – CA1							
Gene	Mean (±SEM) of different treatments		Two-sample t-test *p*-value	Mann-Whitney-U Test *p*-value	Levene's-Test *p-value*	Shapiro-Wilk Test *p-value*	
	SFC- (n=10)	SFC+ (n=10)	SFC+ vs. SFC-	SFC+ vs. SFC-		SFC-	SFC+
Arc	0.92 (±0.04)	0.89 (±0.06)	0.681		0.644	0.414	0.253
cFos	0.85 (±0.09)	0.68 (±0.07)	0.149		0.221	0.243	0.700
Fosl2	1.24 (±0.09)	0.97 (±0.07)	**0.032**		0.503	0.896	0.768
Htr1a	1.09 (±0.06)	1.13 (±0.06)	0.650		0.677	0.981	0.407
Htr2a	1.06 (±0.05)	1.15 (±0.04)	0.164		0.567	0.477	0.288
Npy	0.96 (±0.04)	1.04 (±0.06)	0.262		*0.058*	*0.081*	0.78
Npyr1	0.96 (±0.04)	1.02 (±0.07)	0.475		*0.082*	0.921	0.959
Npyr2	0.96 (±0.04)	1.03 (±0.11)	0.248		0.971	0.517	0.797
Oxtr	1.21 (±0.16)	0.98 (±0.18)		0.280	0.912	**0.038**	**0.041**
Basolateral amygdala							
Gene	Mean (±SEM) of different treatments		Two-sample t-Test *p*-value	Mann-Whitney-U Test *p*-value	Levene's-Test *p-value*	Shapiro-Wilk Test *p-value*	
	SFC- (n=10)	SFC+ (n=10)	SFC+ vs. SFC-	SFC vs. SFC-		SFC-	SFC+
Arc	0.90 (±0.12)	0.85 (±0.06)		0.739	**0.005**	*0.065*	0.138
cFos	0.90 (±0.10)	0.95 (±0.13)		0.971	0.489	0.398	**0.017**
Fosl2	1.06 (±0.08)	1.06 (±0.11)	0.990		0.425	0.345	0.539
Htr1a	0.46 (±0.04)	0.48 (±0.03)	0.777		0.588	0.284	0.884

Htr2a	1.07 (±0.10)	0.98 (±0.06)	0.455		0.397	*0.087*	0.218
Htr2c	0.94 (±0.05)	1.01 (±0.05)	0.401		0.735	*0.076*	*0.071*
Npy	1.00 (±0.06)	0.97 (±0.09)		0.579	0.955	0.198	**0.008**
Npyr1	0.97 (±0.04)	1.10 (±0.05)	*0.069*		0.765	0.991	0.495
Npyr2	0.97 (±0.06)	1.18 (±0.08)	*0.074*		0.301	0.267	0.346
Oxtr	1.10 (±0.09)	1.03 (±0.07)	0.564		0.565	0.872	0.923
Dorsal raphe							
Gene	Mean (±SEM) of different treatments		Two-sample t-Test *p*-value	Mann-Whitney-U Test *p*-value	Levene's-Test *p-value*	Shapiro-Wilk Test *p-value*	
	SFC- (n=10)	SFC+ (n=10)	SFC+ vs. SFC-	SFC+ vs. SFC-		SFC-	SFC+
Arc	1.04 (±0.11)	1.00 (±0.08)	0.806		0.712	0.112	0.863
Avpr1a	1.18 (±0.10)	0.89 (±0.13)	0.107		0.170	0.241	0.266
cFos	1.00 (±0.07)	1.03 (±0.07)	0.764		0.921	0.152	0.637
Fosl2	1.07 (±0.09)	1.03 (±0.06)	0.712		0.156	0.805	*0.094*
Htr1a	1.04 (±0.05)	0.95 (±0.10)	0.425		*0.083*	0.825	0.332
Htr2a	0.94 (±0.15)	1.37 (±0.21)		0.113	**0.046**	*0.058*	*0.089*
Htr2c	0.99 (±0.04)	0.99 (±0.03)	0.795		0.654	0.858	0.201
Npy	1.45 (±0.23)	0.89 (±0.25)		*0.077*	0.631	0.857	**0.031**
Npyr1	1.09 (±0.07)	0.95 (±0.06)		0.222	0.625	**0.031**	0.779
Npyr2	1.08 (±0.08)	0.97 (±0.12)	0.472		0.115	0.420	0.462
Oxtr	1.05 (±0.08)	0.95 (±0.08)	0.442		0.472	0.482	0.400
Tph2	1.27 (±0.12)	1.10 (±0.20)	0.477		*0.077*	0.981	0.350

Table 15: Relative expression data and statistical analysis for all target genes and regions of the prenatal stress study with female animals.

Hypothalamus							
Gene	Mean (±SEM) of different groups				ANOVA (*p*-value)		
	WT C (n=12)	WT PS (n=23)	HET C (n=11)	HET PS (n=27)	Genotype	Treatment	Genotype x Treatment
Avp	1.06 (±0.16)	1.17 (±0.14)	1.17 (±0.15)	1.14 (±0.13)	0.810	0.809	0.647
Avpr1a	0.95	1.01	0.99	1.02	0.755	0.571	0.868

	(±0.06)	(±0.06)	(±0.08)	(±0.07)			
Htr1a	0.99 (±0.08)	1,03 (±0.06)	1.03 (±0.10)	1.07 (±0.08)	0.655	0.624	0.983
Htr2a	1.01 (±0.07)	1.01 (±0.06)	1.07 (±0.12)	1.00 (±0.08)	0.810	0.721	0.718
Htr2c	1,01 (±0,10)	1.07 (±0.08)	1.06 (±0.14)	1.08 (±0.10)	0.801	0.701	0.840
Oxt	1,06 (±0.10)	1.09 (±0.08)	1.04 (±0.09)	1.07 (±0.10)	0.842	0.783	0.973
Oxtr	1,15 (±0.20)	1,11 (±0.06)	1,18 (±0.20)	1.14 (±0.13)	0.442	0.320	0.912
Hippocampus							
	Mean (±SEM) of different groups				ANOVA (*p*-value)		
	WT C (n=12)	WT PS (n=23)	HET C (n=11)	HET PS (n=27)	Genotype	Treatment	Genotype x Treatment
Avp	0.95 (±0.21)	1.27 (±0.23)	2.62 (±0.73)	0.90 (±0.16)	**0.045**	**0.034**	**0.002**
Avpr1a	0.97 (±0.10)	1.28 (±0.23)	1.03 (±0.08)	1.07 (±0.10)	0.922	0.413	0.706
Htr1a	1.15 (±0.07)	1.18 (±0.05)	1.18 (±0.11)	1.15 (±0.56)	0.985	0.982	0.664
Htr2a	1.09 (±0.05)	1.09 (±0.02)	1.07 (±0.06)	1.08 (±0.03)	0.710	0.760	0.840
Htr2c	1.09 (±0.09)	1.07 (±0.04)	1.00 (±0.06)	1.12 (±0.04)	0.717	0.393	0.238
Oxtr	1.17 (±0.09)	1.37 (±0.08)	1.18 (±0.08)	1.33 (±0.06)	0.854	**0.043**	0.758
Amygdala							
	Mean (±SEM) of different groups				ANOVA (*p*-value)		
	WT C (n=12)	WT PS (n=23)	HET C (n=11)	HET PS (n=27)	Genotype	Treatment	Genotype x Treatment
Avpr1a	0.96 (±0.07)	1.02 (±0.05)	0.77 (±0.08)	0.96 (±0.04)	**0.040**	**0.046**	0.249
Htr1a	0.97 (±0.054)	1.01 (±0.04)	0.91 (±0.07)	0.99 (±0.04)	0.359	0.240	0.673
Htr2a	0.97 (±0.06)	1.02 (±0.03)	0.94 (±0.07)	0.97 (±0.04)	0.399	0.327	0.785
Htr2c	0.97 (±0.08)	1.06 (±0.05)	0.94 (±0.11)	1,00 (±0.06)	0.518	0.314	0.868
Oxtr	0.94 (±0.08)	1.02 (±0.03)	0.89 (±0.11)	0.96 (±0.06)	0.442	0.320	0.912
Raphe							
	Mean (±SEM) of different groups				ANOVA (*p*-value)		
	WT C (n=12)	WT PS (n=23)	HET C (n=11)	HET PS (n=27)	Genotype	Treatment	Genotype x Treatment
Avpr1a	1.00 (±0.08)	1.13 (±0.05)	0.88 (±0.09)	1.06 (±0.05)	0.164	**0.025**	0.734
Htr1a	1.08 (±0.08)	1.14 (±0.06)	0.85 (±0.11)	1.07 (±0.06)	*0.059*	*0.069*	0.356
Htr2a	1.03 (±0.07)	1.11 (±0.04)	0.91 (±0.10)	0.99 (±0.05)	*0.085*	0.213	0.951
Htr2c	1.02 (±0.07)	1.14 (±0.06)	0.96 (±0.10)	1.04 (±0.08)	0.327	0.230	0.779

Oxtr	1.05 (±0.10)	1.28 (±0.09)	0.86 (0.12)	1.11 (±0.11)	0.153	*0.052*	0.912
Tph2	1.10 (±0.08)	1.15 (±0.05)	0.87 (±0.11)	0.99 (±0.05)	**0.003**	0.224	0.557

Table 16: Relative expression data and statistical analysis for all target genes and regions of the prenatal stress study with female animals taking results of the 3-chamber sociability test into account.

Hypothalamus									
Gene	Mean (±SEM) of different groups						ANOVA (*p*-value)		
	WT C (n=12)	WT PS social (n=13)	WT PS unsocial (n=10)	HET C (n=11)	HET PS social (n=14)	HET PS unsocial (n=13)	Genotype	Social	Genotype x Social
Avp	1.06 (±0.16)	1.28 (±0.23)	1.04 (±0.11)	1.17 (±0.16)	1.23 (±0.18)	1.04 (±0.17)	0.882	0.468	0.904
Avpr1a	0.95 (±0.06)	1.02 (±0.23)	0.99 (±0.04)	0.99 (±0.08)	1.07 (±0.11)	0.96 (±0.06)	0.786	0.602	0.876
Htr1a	0.99 (±0.08)	1.08 (±0.10)	0.97 (±0.06)	1.03 (±0.10)	1.13 (±0.12)	1.01 (±0.09)	0.609	0.453	1.000
Htr2a	1.01 (±0.07)	1.00 (±0.09)	1.02 (±0.08)	1.07 (±0.12)	1.10 (±0.13)	0.90 (±0.05)	0.930	0.634	0.516
Htr2c	1.00 (±0.10)	1.12 (±0.13)	1.01 (±0.08)	1.06 (±0.14)	1.16 (±0.15)	0.98 (±0.11)	0.502	0.589	0.644
Oxt	1.06 (±0.10)	1.13 (±0.14)	1.06 (±0.08)	1.05 (±0.09)	1.18 (±0.17)	0.96 (±0.11)	0.823	0.479	0.825
Oxtr	1.12 (±0.18)	1.21 (±0.18)	0.99 (±0.10)	1.18 (±0.20)	1.26 (±0.20)	1.02 (±0.16)	0.813	0.425	0.998
Hippocampus									
Gene	Mean (±SEM) of different groups						ANOVA (*p*-value)		
	WT C (n=12)	WT PS social (n=13)	WT PS unsocial (n=10)	HET C (n=11)	HET PS social (n=14)	HET PS unsocial (n=13)	Genotype	Social	Genotype x Social
Avpr1a	0.97 (±0.10)	1.13 (±0.17)	1.07 (±0.07)	1.03 (±0.08)	0.93 (±0.11)	1.20 (±0.17)	0.954	0.555	0.450
Htr1a	1.15 (±0.07)	1.17 (±0.05)	1.20 (±0.11)	1.18 (±0.11)	1.22 (±0.09)	1.07 (±0.07)	0.859	0.752	0.528
Htr2a	1.09 (±0.05)	1.12 (±0.02)	1.05 (±0.03)	1.07 (±0.06)	1.09 (±0.05)	1.08 (±0.03)	0.807	0.638	0.772
Htr2c	1.09 (±0.09)	1.02 (±0.06)	1.14 (±0.07)	1.00 (±0.06)	1.10 (±0.05)	1.15 (±0.07)	0.961	0.332	0.436
Oxtr	1.17 (±0.09)	1.38 (±0.13)	1.37 (±0.05)	1.18 (±0.08)	1.31 (±0.07)	1.36 (±0.08)	0.770	0.134	0.913
Amygdala									
Gene	Mean (±SEM) of different groups						ANOVA (*p*-value)		
	WT C (n=12)	WT PS social (n=13)	WT PS unsocial (n=10)	HET C (n=11)	HET PS social (n=14)	HET PS unsocial (n=13)	Genotype	Social	Genotype x Social
Avpr1a	0.96 (±0.07)	1.01 (±0.07)	1.02 (±0.07)	0.77 (±0.08)	0.89 (±0.06)	1.03 (±0.07)	*0.075*	*0.084*	0.355
Htr1a	0.97 (±0.05)	1.04 (±0.06)	0.98 (±0.04)	0.91 (±0.07)	0.94 (±0.04)	1.04 (±0.06)	0.451	0.479	0.328
Htr2a	0.97 (±0.06)	1.07 (±0.03)	0.97 (±0.04)	0.94 (±0.07)	0.95 (±0.05)	1.00 (±0.06)	0.371	0.587	0.406
Htr2c	0.97 (±0.08)	1.10 (±0.07)	1.00 (±0.07)	0.94 (±0.11)	0.97 (±0.07)	1.03 (±0.10)	0.502	0.589	0.644

Oxtr	0.94 (±0.08)	1.09 (±0.07)	0.94 (±0.06)	0.89 (±0.11)	0.91 (±0.06)	1.01 (±0.12)	0.451	0.602	0.353
Raphe									
Gene	Mean (±SEM) of different groups						ANOVA (*p*-value)		
	WT C (n=12)	WT PS social (n=13)	WT PS unsocial (n=10)	HET C (n=11)	HET PS social (n=14)	HET PS unsocial (n=13)	Genotype	Social	Genotype x Social
Avpr1a	1.00 (±0.08)	1.06 (±0.07)	1.22 (±0.07)	0.88 (±0.09)	1.06 (±0.07)	1.05 (±0.09)	0.142	*0.050*	0.547
Htr1a	1.08 (±0.08)	1.12 (±0.08)	1.19 (±0.08)	0.85 (±0.11)	1.11 (±0.08)	1.02 (±0.09)	*0.082*	0.185	0.438
Htr2a	1.03 (±0.07)	1.09 (±0.06)	1.13 (±0.06)	0.91 (±0.10)	1.02 (±0.07)	0.97 (±0.08)	*0.066*	0.457	0.826
Htr2c	1.02 (±0.07)	1.11 (±0.09)	1.19 (±0.10)	0.96 (±0.10)	1.09 (±0.11)	0.98 (±0.11)	0.230	0.471	0.590
Oxtr	1.05 (±0.10)	1.24 (±0.14)	1.32 (±0.13)	0.86 (0.12)	1.20 (±0.13)	1.02 (±0.17)	0.123	0.136	0.620
Tph2	1.10 (±0.08)	1.15 (±0.07)	1.15 (±0.08)	0.87 (±0.11)	1.04 (±0.04)	0.93 (±0.08)	**0.003**	0.355	0.608

Table 17: Relative expression data and statistical analysis for all target genes and regions of the prenatal stress study with male animals.

Hypothalamus							
Gene	Mean (±SEM) of different groups				ANOVA (*p*-value)		
	WT C (n=9)	WT PS (n=7)	WT C (n=9)	WT PS (n=7)	Genotype	Treatment	Genotype x Treatment
Avp	1.37 (±0.21)	1.54 (±0.47)	0.95 (±0.09)	1.25 (±0.30)	0.177	0.362	0.812
Avpr1a	1.05 (±0.08)	1.23 (±0.25)	0.96 (±0.07)	1.12 (±0.10)	0.410	0.157	0.962
Htr1a	1.06 (±0.09)	1.16 (±0.14)	1.03 (±0.07)	1.02 (±0.11)	0.419	0.679	0.607
Htr2a	1.07 (±0.08)	1.10 (±0.15)	0.97 (±0.04)	1.04 (±0.09)	0.339	0.592	0.834
Htr2c	1.05 (±0.07)	1.20 (±0.26)	0.98 (±0.05)	1.02 (±0.08)	0.235	0.368	0.601
Npy	1.23 (±0.16)	1.14 (±0.15)	1.03 (±0.11)	1.07 (±0.12)	0.340	0.827	0.648
Npyr1	1.10 (±0.12)	1.21 (±0.27)	0.96 (±0.04)	1.08 (±0.12)	0.301	0.361	0.987
Npyr2	1.10 (±0.08)	1.13 (±0.14)	0.99 (±0.05)	0.99 (±0.08)	0.138	0.904	0.858
Oxt	1.23 (±0.14)	1.35 (±0.22)	0.95 (±0.07)	1.11 (±0.18)	0.101	0.378	0.878
Oxtr	1.27 (±0.13)	1.51 (±0.58)	0.99 (±0.08)	1.10 (±0.17)	0.142	0.448	0.777
Hippocampus							
	Mean (±SEM) of different groups				ANOVA (*p*-value)		
	WT C (n=9)	WT PS (n=7)	WT C (n=9)	WT PS (n=7)	Genotype	Treatment	Genotype x Treatment
Avpr1a	0.95 (±0.06)	1.05 (±0.08)	1.03 (±0.05)	1.05 (±0.06)	0.552	0.378	0.556
Htr1a	1.02 (±0.07)	1.03 (±0.07)	1.03 (±0.05)	0.98 (±0.04)	0.747	0.378	0.591

Htr2a	1.12 (±0.11)	1.11 (±0.13)	1.02 (±0.06)	0.95 (±0.07)	0.129	0.731	0.704
Htr2c	1.02 (±0.07)	0.97 (±0.04)	1.04 (±0.04)	0.99 (±0.04)	0.669	0.619	0.909
Npy	1.05 (±0.09)	0.96 (±0.04)	1.03 (±0.05)	1.00 (±0.04)	0.901	0.312	0.656
Npyr1	0.94 (±0.04)	1.10 (±0.07)	1.00 (±0.04)	1.03 (±0.05)	0.982	0.299	0.186
Npyr2	1.08 (±0.17)	1.13 (±0.17)	1.14 (±0.09)	0.99 (±0.11)	0.785	*0.071*	0.474
Oxtr	0.95 (±0.06)	1.05 (±0.08)	1.03 (±0.05)	1.05 (±0.06)	0.552	0.700	0.556
Amygdala							
	Mean (±SEM) of different groups				ANOVA (*p*-value)		
	WT C (n=9)	WT PS (n=7)	WT C (n=9)	WT PS (n=7)	Genotype	Treatment	Genotype x Treatment
Avpr1a	1.02 (±0.10)	1.03 (±0.12)	1.06 (±0.06)	1.02 (±0.07)	0.886	0.893	0.765
Htr1a	0.94 (±0.09)	1.14 (±0.14)	1.02 (±0.06)	1.08 (±0.7)	0.882	0.152	0.456
Htr2a	0.96 (±0.05)	1.10 (±0.09)	1.01 (±0.06)	1.04 (±0.06)	0.965	0.209	0.387
Htr2c	0.96 (±0.06)	1.09 (±0.13)	1.03 (±0.08)	1.06 (±0.07)	0.852	0.343	0.594
Npy	0.96 (±0.05)	1.06 (±0.06)	1.00 (±0.05)	1.05 (±0.04)	0.751	0.166	0.683
Npyr1	0.93 (±0.07)	1.12 (±0.12)	1.02 (±0.07)	1.09 (±0.06)	0.763	0.118	0.450
Npyr2	0.92 (±0.07)	1.13 (±0.11)	0.99 (±0.04)	1.09 (±0.06)	0.895	**0.029**	0.453
Oxtr	0.83 (±0.08)	1.23 (±0.28)	1.07 (±0.11)	1.26 (±0.14)	0.372	*0.062*	0.472
Raphe							
	Mean (±SEM) of different groups				ANOVA (*p*-value)		
	WT C (n=9)	WT PS (n=7)	WT C (n=9)	WT PS (n=7)	Genotype	Treatment	Genotype x Treatment
Avpr1a	1.01 (±0.16)	1.37 (±0.15)	1.00 (±0.08)	1.03 (±0.09)	0.148	0.102	0.168
Htr1a	1.10 (±0.15)	1.32 (±0.23)	1.02 (±0.09)	1.000 (±0.07)	0.117	0.434	0.328
Htr2a	1.04 (±0.13)	1.29 (±0.17)	0.98 (±0.06)	1.01 (±0.07)	*0.089*	0.156	0.294
Htr2c	1.22 (±0.17)	1.43 (±0.26)	0.95 (±0.08)	0.97 (±0.06)	**0.006**	0.347	0.452
Npy	1.08 (±0.08)	1.07 (±0.08)	1.05 (±0.07)	0.95 (±0.06)	0.308	0.482	0.534
Npyr1	1.16 (±0.16)	1.32 (±0.23)	0.98 (±0.07)	0.99 (±0.08)	*0.047*	0.497	0.551
Npyr2	1.09 (±0.15)	1.26 (±0.20)	1.03 (±0.08)	0.98 (±0.06)	0.142	0.577	0.311
Oxtr	1.33 (±0.35)	1.81 (±0.36)	1.07 (±0.15)	1.01 (±0.16)	**0.030**	0.392	0.263
Tph2	1.00 (±0.10)	1.24 (±0.05)	1.00 (±0.05)	0.98 (±0.06)	*0.071*	0.124	*0.068*

Table 18: Relative expression data and statistical analysis for all target genes and regions of the follow-up study with male animals of all 5-Htt genotypes.

Hypothalamus					
Gene	Mean (±SEM) of different groups			ANOVA (*p*-value)	Bonferroni Post hoc
	WT (n=16)	HET (n=15)	KO (n=16)	Genotype	Genotype
Avp	1,06 (±0,13)	1,20 (±0,11)	1,22 (±0,12)	0,830	
Avpr1a	1,06 (±0,07)	0,96 (±0,04)	1,04 (±0,06)	0,403	
Htr1a	1,02 (±0,06)	0,97 (±0,04)	1,08 (±0,05)	0,243	
Htr2a	0,95 (±0,05)	0,93 (±0,03)	0,94 (±0,04)	0,384	
Htr2c	1,07 (±0,07)	0, 98 (±0,06)	1,01 (±0,05)	0,728	
Npy	1,18 (±0,11)	1,00 (±0,09)	0,99 (±0,08)	0,386	
Npyr1	1,07 (±0,08)	0,98 (±0,05)	1,02 (±0,05)	0,529	
Npyr2	1,00 (±0,05)	1,00 (±0,06)	1,09 (±0,03)	0,211	
Oxt	1,04 (±0,09)	1,13 (±0,08)	1,19 (±0,12)	0,546	
Oxtr	1,11 (±0,13)	0,97 (±0,08)	1,11 (±0,08)	0,511	
Hippocampus					
	Mean (±SEM) of different groups			ANOVA (*p*-value)	Bonferroni Post hoc
	WT (n=16)	HET (n=16)	KO (n=16)	Genotype	Genotype
Avpr1a	1,03 (±0,91)	1,24 (±0,24)	1,03 (±0,08)	0,549	
Htr1a	0,93 (±0,87)	1,06 (±0,11)	1,17 (±0,05)	0,155	
Htr2a	0,95 (±0,80)	1,02 (±0,06)	1,13 (±0,05)	0,123	
Htr2c	1,00 (±0,74)	1,05 (±0,09)	1,06 (±0,05)	0,820	
Npy	1,03 (±0,23)	0,99 (±0,03)	0,99 (±0,02)	0,505	
Npyr1	0,96 (±0,62)	1,05 (±0,09)	1,08 (±0,05)	0,440	
Npyr2	0,89 (±0,66)	1,07 (±0,12)	1,19 (±0,07)	*0,056*	*0,055*
Oxtr	0,96 (±1,00)	1,07 (±0,14)	1,21 (±0,07)	0,259	
Amygdala					
	Mean (±SEM) of different groups			ANOVA (*p*-value)	Bonferroni Post hoc
	WT (n=16)	HET (n=16)	KO (n=16)	Genotype	Genotype
Avpr1a	0,73 (±0,11)	0,77 (±0,08)	0,65 (±0,07)	0,388	
Htr1a	1,10	1,06	1,16	0,948	

	(±0,12)	(±0,09)	(±0,09)		
Htr2a	1,12 (±0,11)	0,99 (±0,08)	1,20 (±0,10)	0,505	
Htr2c	1,07 (±0,12)	0,98 (±0,10)	1,17 (±0,11)	0,370	
Npy	1,03 (±0,03)	1,02 (±0,04)	1,00 (±0,03)	0,496	
Npyr1	1,14 (±0,12)	1,02 (±0,09)	1,15 (±0,10)	0,717	
Npyr2	1,10 (±0,10)	1,01 (±0,07)	1,07 (±0,08)	0,736	
Oxtr	1,25 (±0,20)	1,08 (±0,16)	1,35 (±0,17)	0,576	
Raphe					
	Mean (±SEM) of different groups			ANOVA (*p*-value)	Bonferroni Post hoc
	WT (n=16)	HET (n=16)	KO (n=16)	Genotype	Genotype
Avpr1a	1,02 (±0, 04)	0,94 (±0,05)	1,10 (±0,03)	**0,025**	**0,021**
Htr1a	1,04 (±0,05)	0,97 (±0,07)	1,09 (±0,05)	0,335	
Htr2a	1,06 (±0,05)	0,95 (±0,06)	1,06 (±0,04)	0,215	
Htr2c	1,06 (±0,05)	0,96 (±0,09)	1,11 (±0,05)	0,252	
Npy	1,02 (±0,06)	1,01 (±0,07)	1,07 (±0,09)	0,827	
Npyr1	1,02 (±0,07)	0,97 (±0,08)	1,12 (±0,06)	0,277	
Npyr2	1,04 (±0,04)	0,93 (±0,06)	1,08 (±0,04)	*0,068*	*0,077*
Oxtr	1,02 (±0,05)	0,95 (±0,08)	1,16 (±0,05)	*0,063*	*0,064*
Tph2	1,04 (±0,02)	1,03 (±0,05)	0,96 (±0,03)	0,222	

Table 19: Relative expression data and statistical analysis for all target genes and regions of the follow-up study with female animals of all 5-Htt genotypes and with high or low estrous levels.

	Hypothalamus								
Gene	Mean (±SEM) of different groups						ANOVA (*p*-value)		
	High estrous			Low estrous					
	WT (n=15)	HET (n=15)	KO (n=15)	WT (n=15)	HET (n=15)	KO (n=15)	Genotype	Estrous	Genotype x Estrous
Avp	1,18 (±0,14)	1,41 (±0,18)	1,46 (±0,15)	1,09 (±0,18)	1,20 (±0,13)	1,10 (±0,15)	0,508	*0,088*	0,698
Avpr1a	1,09 (±0,09)	1,14 (±0,08)	1,03 (±0,04)	1,03 (±0,08)	1,05 (±0,05)	0,96 (±0,05)	0,363	0,229	0,979
Oxt	1,20 (±0,11)	1,37 (±0,14)	1,39 (±0,10)	1,09 (±0,14)	1,16 (±0,11)	1,04 (±0,09)	0,607	**0,022**	0,596
Oxtr	0,99 (±0,10)	0,94 (±0,08)	1,07 (±0,08)	1,15 (±0,09)	1,01 (±0,09)	1,17 (±0,09)	0,250	0,118	0,866
	Hippocampus								
	Mean (±SEM) of different groups						ANOVA (*p*-value)		
	High estrous			Low estrous					

	WT (n=15)	HET (n=15)	KO (n=15)	WT (n=15)	HET (n=15)	KO (n=15)	Genotype	Estrous	Genotype x Estrous
Avp	1,06 (±0,18)	1,15 (±0,31)	2,08 (±1,15)	2,98 (±1,48)	1,96 (±0,69)	3,51 (±1,17)	0,447	*0,090*	0,848
Oxtr	0,94 (±0,11)	0,99 (±0,10)	0,91 (±0,12)	1,30 (±0,14)	1,19 (±0,13)	1,28 (±0,13)	0,960	**0,003**	0,762

6.2 List of figures

6.3 List of tables

Affidavit

I hereby confirm that my thesis entitled “Fear and anxiety disorders – interaction of AVP and OXT brain systems with the serotonergic system” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.
Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

_____ _____

Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Furcht und Angsterkrankungen – Interaktion von AVP und OXT Gehirnsystemen mit dem serotonergen System“ eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

_____ _____

Ort, Datum Unterschrift

Curriculum Vitae

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