Involvement of neuropeptide systems in social and cued fear conditioning in rats

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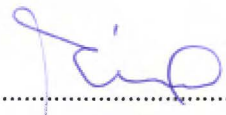
unter Anleitung von

Prof. Dr. rer. nat. Inga D. Neumann

Author's declaration
Herewith, I declare that this thesis is my own work and I did not make use of any other sources and auxiliary means besides those listed in the bibliography.



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Marianella Masís Calvo
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“Above all, don’t fear difficult moments. The best comes from them.”

– Rita Levi-Montalcini

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Summary

Summary

Fear and pathological avoidance of specific situations is a recurrent symptom of patients with psychiatric disorders, for example, patients with social anxiety disorder patients (SAD) avoid social situations, while, Post-Traumatic Stress Disorder (PTSD), avoid environments related to intrusive memories. The present dissertation's main goal was to study the neurobiological underpinnings of fear and anxiety-related behaviors that help us to understand better such psychiatric disorders. For that purpose, two projects comprising fear conditioning models in laboratory rats were developed. In **Part I** of the thesis, I have been entrusted with implementing a neurogenetic animal model of social fear conditioning (SFC) in three rat lines, which differ in their constitutive anxiety levels. In such approach, I combined the equivalent aspects of conditioning events (SFC) and the behavioral inhibition constructs (High anxiety-related behavior in rats) for study the development of SAD. These lines were selected and bred based upon their unconditioned anxiety-related behaviors in the elevated plus-maze test resulting in two rat lines of high anxiety behavior (HAB) and low anxiety behavior (LAB). First, I successfully standardized the model in non-selected rats (NAB) rats, which showed social fear memory in short-term fashion (up to 6 h). Afterward, different SFC experiments conducted in HAB and LAB rats revealed that both opposite lines were more vulnerable to acquire for long-term (24 h) social fear than NAB rats. Notably, HAB rats showed individual recognition of the stimulus associated with social fear acquisition. This observation led to the hypothesis that an increase in AVP (well-known enhancer of social recognition) may have mediated this long-term social recognition in HAB rats, as this line has a single-nucleotide polymorphism in the AVP promotor, that results in a higher availability of this nonapeptide. Therefore, I tested blocking the V1a receptor (V1aR-A) or giving exogenous AVP either centrally or locally in the LS to HAB, LAB, and NAB rats. I found that HAB and LAB rats represent the extreme ends of an inverted U-shaped curve in terms of their response to AVP in the consolidation of social-fear memory (*i.e.*, HAB/V1aR-A rats and LAB/AVP rats, both reduce social fear consolidation). Also, only NAB/SFC$^-$ rats showed enhanced social discrimination after i.c.v. treatment with AVP and failed promoted social fear after 24 h. In contrast, infusing the same treatments in the LS of HAB and LAB rats did not reveal significant effects, with the HAB rats losing their social discrimination ability. The lack of effects observed within the LS can be due to time-dependent differences in the ability of AVP to exert their maximum effects or to a weaker involvement of LS in memory consolidation. The previous results showed that AVP contributes only partially to the social fear consolidation, suggesting that both lines can share alternative mechanisms; however, it does not explain NAB rats' resilience.

Memory consolidation can be either enhanced or impaired by GCs. Interestingly, both lines (HAB and LAB) showed dysregulation of the stress response. Therefore, I investigated the impact of GCs on the long-term consolidation of social fear memory in HAB, LAB, and NAB rats. First, I compared the plasma Cort levels between lines (HAB *vs* NAB) and within lines (SFC^+ *vs*. SFC^-) during the different SFC phases. LAB rats were excluded from this experiment due to limited reproductive rates. Based on the higher basal Cort levels in HAB compared to NAB rats, my second approach was to assess the effect of blocking Cort signaling by Metyrapone treatment (Met, an inhibitor of Cort synthesis) in HAB and LAB rats before the acquisition of social fear on memory consolidation. Indeed, Met impaired the social fear consolidation in HAB and LAB rats. Finally, I assessed the effects of Cort (i.p. or i.c.v.) in NAB rats to test the hypothesis that higher Cort in NAB rats may prolong the social fear memory from 6 to 24 h. Systemic and central Cort administration did not show significant differences compared with the Veh groups, perhaps because the sample size was too small in both experiments. However, NAB ras showed social fear after 24 h after stress due to the injection. Altogether, these findings fit with the Yerkes-Dobson law regarding their memory performance. Finally, I quantified the number of c-Fos positive cells in the amygdala and hippocampus, which are relevant brain regions related to social fear discrimination in HAB and NAB. In this regard, only the activation of the CA2/3 areas was significantly reduced in conditioned NAB rats compared to the unconditioned group during the social fear discrimination test, 6 hours after the social fear acquisition. In conclusion, both extreme phenotypes (HAB and LAB) allowed the study of different risk factors for acquiring social-related trauma (*i.e.*, AVP or GC), while NAB rats represent the opportunity to study the resilience mechanisms.

In **Part II** of the thesis, I aimed to study the effects of the neuropeptide S (NPS) system in female rats. The NPS system has been identified as an important neuromodulator involved in fear, anxiety, and stress response. However, our current knowledge about how the NPS and its receptor (NPSR) is regulated in females is restricted to few studies. Thus, to better understand the female response and the NPS system, I included female rats in different reproductive states (*i.e.*, virgin *vs*. lactating females). My first approach was to describe the mRNA levels of NPS and NPSR in stress-related brain regions. Q-PCR analysis revealed an upregulation of the NPS levels but not of its receptor during lactation within the locus coeruleus, paraventricular nuclei, and amygdala compared to the virgin group. Moreover, I evaluated if this upregulation putative contribute to the cued fear response conditioning. The day after fear conditioning, all animals where centrally infused

with NPS, its antagonist or vehicle solution before fear extinction trials. Based on their estrus cycle, virgin females were additionally split into two groups of low and high estradiol levels for the statistical analysis. All females learned the cued fear independent of their reproductive cycle. In contrast, during the fear extinction, significant effects of time (CS presentation), treatment, and a strong reproductive status trend were found. Central NPS infusions reduced the freezing response in lactating rats during the cued fear extinction training. Virgins with lower estradiol levels were susceptible to the NPS antagonist effects, which delayed the cued fear extinction processes. In contrast, virgins with higher estradiol levels did not show treatment effects. Additionally, I measured the corticosterone (Cort), Oxytocin (OXT), and NPS levels in trunk-blood samples after the same treatment described above. I found an increase in Cort levels in virgins treated with NPS, but no effects in lactating female rats. Furthermore, OXT levels were increased in both groups, and no between-group differences in the NPS were observed. Furthermore, I confirmed the effects of NPS on the Cort levels in virgin rats monitored with a jugular-vein catheter at different time-points post-infusion. In contrast, lactating females infused with the NPS antagonist showed no differences compared with the Veh group.

In conclusion, *NPS* expression was shown to be differentially regulated according to the reproductive states in relevant stress-related regions. Moreover, in LE virgin and lactating females, the NPS treatment facilitates the fear extinction, whereas its antagonist delayed it. In contrast, HE females showed the lowest freezing levels, suggesting a protective effect of estradiol against traumatic experiences, suggesting that NPS's anxiolytics effects are also sensitive to sex hormones variations. However, the exact mechanism by which estradiol interacts with the NPS still needs to be elucidated. Furthermore, I showed that peripheral stress parameters, such as Cort and OXT, positively correlate with NPS administration in virgin females, while in lactating females, only OXT levels were increased. Altogether, these findings highlight the NPS relevance as a potential treatment for stress-related disorders and as a modulator of maternal behavior.

List of abbreviations

A

ACTH adrenocorticotropic hormone

AMY amygdala

ANOVA Analysis of variance

ANS autonomous nervous system

AP

AVP arginine vasopressin

B

B# basal sample

BI behavioral inhibition trait

BLA basolateral amygdala

BNST bed nucleus stria terminalis

BP blood pressure

C

CA *cornu ammonis*

CA1 *cornu ammonis 1*

CA2/3 *cornu ammonis2 and 3 regions*

CEA central amygdala

CBT cognitive behavioral therapy

cDNA complementary DNA

CFC cued fear conditioning

CNS central nervous system

Cort corticosterone

CR conditioned response

CRF corticotropin-releasing factor

CRFR corticotropin-releasing factor receptor

CS conditioned stimulus

CSF cerebrospinal fluid

CYP11B1 steroid 11β-monooxygenase

CYP11B2 steroid 18-hydroxylase

D

DAB 3,3'-diaminobenzidine

df degrees of freedom

DG dental gyrus

DMH dorsomedial hypothalamic nucleus

DMN dorsal motor nucleusof the vagus

DNA deoxyribonucleic acid

DR discrimination ratio

DSM Diagnostic Statistical Manual for Mental disorders

DV

E

EDTA ethylendiamintetraacetic Acid

e.g. exempli gratia

ELISA enzyme-linked immunosorbent assay

EP elevated platform

EPM elevated plus-maze

F

FDA Food and Drug Administration/USA

*f*MRI functional magnetic resonance imaging

FSH follicle stimulating hormone

G

GABA γ-aminobutyric acid

GAPDH glyceraldehyde-3-phosphate dehydrogenase

GC glucocorticoid

Glu glutamate

GPCR G protein-coupled receptor

GR glucocorticoid receptor

H

HAB high anxiety-related behavior

HC home cage group

HE high estradiol

HP hippocampus

HPA hypothalamic-pituitary-adrenal

HT Hargreaves plantar test

I

icv intracerebroventricular

i.e Latin "*id est", " that is"*

IF infusion

IL infralimbic cortex

i.p. intraperitoneal

J

JVC jugular vein catheter

L

LAB low anxiety-related behavior

LC locus coeruleus

LDB light-dark box

LE low estradiol

LH luteinizing hormone

LHP lateral hypothalamic nuclei

LPS lipopolysaccharide

LS lateral septum

M

mA milliAmpere

MEA medial amygdala

Met metyrapone

mPOA medial preoptic area

MR mineralocorticoid receptor

mRNA messenger RNA

N

NA noradrenaline

NAB rats non-selected for anxiety-like behavior

NAc nucleus accumbens

NC new cage

NPS neuropeptide S

NPSR neuropeptide S receptor

NPSR-A neuropeptide S receptor antagonist

NTS nucleus tractus solitarius

O

OA open arm

OF open field

OXT oxytocin

P

P# pregnancy day

PAG periaqueductal gray

PBN parabrachial nucleus

PBS phosphate buffered saline

PCR polymerase chain reaction

PFA paraformaldehyde

PFC prefrontal cortex

PND postnatal day

PR preference ratio in SPM

PTSD post-traumatic stress disorders

PVN paraventricular nucleus of the hypothalamus

Q

qPCR quantitative PCR

R

RNA ribonucleic acid

RPC caudal reticulopontine nucleus

rpm revolutions per minute

R-SFC reference SFC^+ group

RT room temperature

S

SAD social anxiety disorders

SCN suprachiasmatic nucleus

SEM standard error of the mean

SFC social fear conditioning

SFC^+ social fear conditioned

SFC^- social fear unconditioned

SNS sympathetic nervous system

SNP single nucleotide polymorphism

SON supraoptic nucleus

SPM social preference modified

SPT social preference test

SSRI selective serotonin reuptake inhibitor

U

UR unconditioned response

US unconditioned stimulus

UV ultrasonic vocalizations

V

V1a vasopressin receptor a

V1a-A vasopressin receptor antagonist

V1b vasopressin receptor b

Veh vehicle

vs versus

Introduction

1. Introduction

Fear and anxiety-related behaviors are conserved defensive responses among animals, improving their survival and fitness. As my thesis title suggests, "*Involvement of neuropeptide systems in social and cued fear conditioning in rats*"; I worked on two animal models that served to study those responses. First, I used the Social fear conditioning (SFC) to study the role of vasopressin (AVP) and glucocorticoids (GC) in the consolidation of the social fear memory in male rats. In the second part of the thesis, I mainly focused on the effects of the neuropeptide S (NPS) in female rats using the cued fear conditioning (CFC). Both models shared the use of conditioning procedures to study the fear and anxiety-related behaviors in rats. Therefore, in the first sections of this introduction, I will provide a general background for each behavioral response, the learning and memory processes, and the neurocircuitries related to the fear and anxiety-related responses.

Furthermore, in each model, I addressed not only the role of different neuropeptides (*i.e.,* neurotransmitter imbalance), but also the effect of other factors related to the susceptibility to develop pathological states (*e.g.,* stress imbalance or female-specific risks). Thus, in the following sections, I include an outline of some of the psychiatric disorders and the risk factors related to pathological fear and anxiety responses in humans. Besides, I will describe in more detail how to investigate these aspects using animal models. Finally, in the aims section, I will explain how those factors were combined to study specific goals. I hope this brief overview of the introduction guided the reader through the different topics included here.

1.1 **Fear and anxiety responses**

Distinguishing between fear and anxiety is not a trivial task, however, some authors suggest that a clear distinction is possible based on the behavioral patterns (including time courses and intensities), pharmacological response and the etiology between both cons. Therefore, fear is conventionally described as a response to a well-defined threat, while anxiety state (or anxiety-related behaviors) is driven by stimuli related to potential or ambiguous threats (Blanchard and Blanchard, 2008; LeDoux and Pine, 2016). Both responses elicit a behavioral repertoire that is species-specific (Bolles, 1970). For instance, the presence of a predator elicits fear behaviors in an experimental subject, while the potential presence of the predator (*i.e.*, only its odor) induces more anxiety-related behaviors (Blanchard et al., 2008). Indeed, this differentiation is supported by pharmacological assays where classic anxiolytics alleviate high anxiety states but fail to alleviate panic responses (McNaughton and Zangrossi, 2008). However, this does not mean that fear and

anxiety-related behaviors necessarily excluded from each other. Indeed, both responses can overlap in response to a context where the defensive distance towards a threat gradually varies (Fanselow, 2018). In the next sections, I will describe the fear and anxiety-like repertoire in more detail (*section 1.1.1*), as well as its relationship with the learning and memory processes (*section 1.1.2*). Furthermore, both responses lead to a higher or lower physiological response, preparing the organism to cope with threats. Specific brain networks seem to intervene in these distinct coping strategies (LeDoux and Pine, 2016; Steimer, 2002). Thus, in *section 1.1.3*, I will describe part of the neurocircuitries involved in the regulation of fear and anxiety-like behaviors.

1.1.1 Fear and anxiety-like behaviors in rodents

Fear behaviors: *The three "F´s" … flight-fight-freeze*

Fear responses are often classified into active *versus* passive coping strategies. Active coping occurs when escaping from a threat is possible, to which the autonomic system is associated mainly by sympathetic nervous system activation (SNS, see detailed description in *section 1.4.1*)(Steimer, 2002). These are the so-called "*fight-or-flight*" responses described by Cannon (1920) including increased blood flow to the skeletal muscles, release of glucose from the liver, dilation of bronchi to increase the availability of oxygen, reduced blood flow to the skin and digestive system, and activate adrenal medulla secretion (McCarty, 2016).

Passive coping strategies, such as freezing, are usually provoked by distal threats or when a threat is inescapable. This induces an autonomic inhibition (*i.e.*, hypotension, bradychardia), and activation of the hypothalamic-pituitary-adrenal axis (HPA axis, described in detail in *section 1.4.1*). Coping strategies are determined by multiple factors, for example, the threatening situation, species, sex, age, and individual differences in coping styles (Blanchard and Blanchard, 2008; Gruene et al., 2015; Hashikawa et al., 2018; McNaughton and Corr, 2004; Roelofs, 2017). Due to the setup of the studied animal models here (*see sections 1.4.3 and 1.4.4*), the experimental subject actions are limited (*i.e.*, aggressive behaviors are restricted) or presented an inescapable situation, I focused on freezing behavior. However, fight and flight behaviors are key in the defense against a threat, especially in a social context, described in more detail in the previously published reviews (de Boer et al., 2016; de Boer et al., 2017; Hashikawa et al., 2018; Masis-Calvo et al., 2018).

Freezing is a combination of immobility (except for small respiration-related movements) and tight muscles posture (Fanselow, 1980). It is induced by distal threats, such as predator odors, and visual stimuli (Blanchard, 1997; Fanselow, 1980). In an ecological context, freezing prevents the

detection by predators, because the predator's visual systems are highly sensitive to movement (Bolles, 1970; Fanselow and Ponnusamy, 2008). In the laboratory context, freezing that followed an electric foot shock during a conditioning procedure (see *section 1.1.2*) emerged as a way to deal with an exogenous stimulus during an inescapable situation (Bolles, 1970; Fanselow and Ponnusamy, 2008). From the translational point of view, humans also freeze in response to threats. Indeed, neuroimaging studies indicate similar involvement of brain regions in rodents and humans, controlling freezing responses (see a detailed review in (Roelofs, 2017).

Anxiety-like behaviors: *a conflict between approach and avoidance*

Theoretically, anxiety states (or anxiety-related behaviors) emerge from the expectation of threats due to unprotected situations or ambiguous signals (LeDoux and Pine, 2016; Ohl et al., 2001). Ethological studies found that rodents show an innate drive to explore environments that potentially provide resources. However, at the same time, they fear the novelty and prefer protected areas (Montgomery, 1955). The conflict between approach/avoidance to a novel environment serves as a construct to define the anxiety state of a subject in many unconditioned animal models (*e.g.*, open field, elevated plus maze (EPM), zero-maze, T-maze, dark-light box, and marbel burying test) (Litvin et al., 2008). Over the years, those models proved to have great predictive validity to anxiolytic drugs (Griebel et al., 1997; McNaughton and Zangrossi, 2008).

In most of these tests, spatial and temporal measures reflect the anxiety state of the subject by the preference of anxiogenic versus anxiolytic areas. What defines an anxiogenic area or the opposite relies on its characteristics perceived by the rodents as adverse conditions, such as illumination, height, availability of thigmotaxis, or refuge, among others (Litvin et al., 2008). Hence, a preference for protected areas and avoidance of unprotected ones is interpreted as a high anxiety state, while high exploration of it is considered the opposite. Moreover, the frequency of the so-called risk assessment behaviors, such as stretch-attempt postures, head-dipping, and rearing are also circumscribe a high anxiety state (Blanchard and Blanchard, 2008; Ohl et al., 2001).

1.1.2 Fear and anxiety-related behaviors and their relation with learning and memory processess

Animals face different threats through life, from predators to rival conspecifics that can harm them or disturb their fitness. It is not surprising that a rapid associative learning process of the cues of a threat situation mediates the fear and anxiety-related responses (Fanselow and Ponnusamy, 2008).

Although, these defensive responses especially to cope with predators, need to be displayed as fast as possible, thus, sometimes rely more in pre-programming mechanisms (*i.e.*, genetically determined response or defensive reflexes) or vicarious learning (for instance, follows the reaction of their conspecifics) than associative learning processes (for review see (Blanchard and Blanchard, 2008; LeDoux and Daw, 2018). Herewith, I will review some basic notions of the associative learning and memory processing, especially the classical (*section 1.4.4*) and the passive avoidance conditioning.

Classical conditioning: *an old but gold model*

With this model Pavlov (1927) described the associative learning that enable individuals to extract temporal and causal information from different stimuli (Fanselow, 2018; Rescorla, 1988). This represent a powerful mechanism by which individuals "auto-shape" their behavior to adapt to the environment (reviewed in (Rescorla, 1988). Classical conditioning starts with a natural association between a response (unconditioned response, **UR**) and a stimulus (unconditioned stimulus, **US**). Then, in a process called **acquisition**, a **conditioned stimulus** (**CS**, initially with neutral properties, it can be a tone, light or an odor) is presented in contiguity with an US and in a reliable manner to form an association between them. If the association was successfully learned, the CS elicits the UR without the US, and this is called **conditioned response (CR)**. In this thesis, I implemented the cued fear conditioning (Fig.1 A, see section 1.4.4), in which an electric foot shock is the US and fear expression is measured by freezing time as the UR, that in turn is pairing with a tone (CS).

However, those association memories can be modified, meaning the CR can decrease or disappear over time (spontaneously) or by **extinction training** (repeated non-reinforced presentations). This is considered as a second learning process, called **fear extinction** (Fanselow and Ponnusamy, 2008). Nevertheless, extinction is not flawless and there are different events of fear relapse (Singewald and Holmes, 2019), such as, **spontaneous recovery** (with the passage of time), **external disinhibition** (after including a new stimulus in the extinction context), **reinstatement** (facing the US again), and renewal (when a CS is present in non-extinction contexts) (Bouton and King, 1983; Maren and Holmes, 2016; Rescorla and Heth, 1975). Watson and Rayner (1920) proved the translational value of the pavlovian principles in the "*little Albert*" experiments (Fanselow and Ponnusamy, 2008), although, from the ethical perspective those findings belong to a rather dark chapter of science.

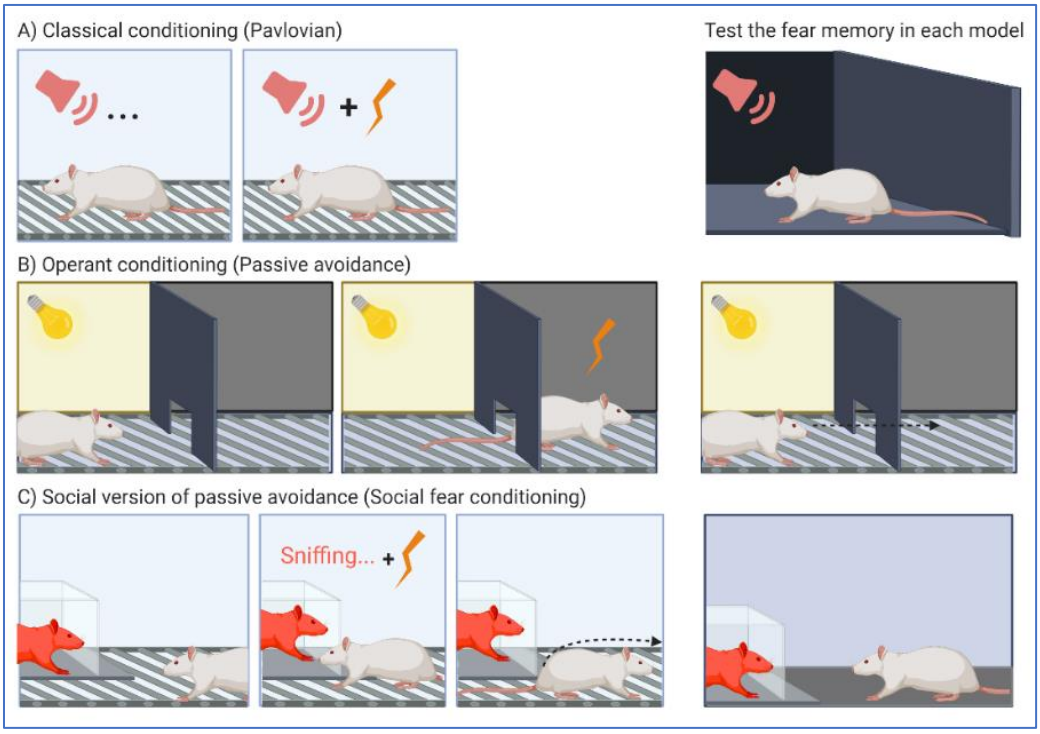


Fig.1 Conditioning paradigms. A) Classical conditioning. In this example a tone (CS) is paired with an electric foot shock (US) which promoted fear (UR). Afterwards, the tone alone evoked the fear (CR). Then, tested by the CS presentations alone. B) Passive avoidance (Step-through), a rat preferred a dark *vs*. a light box, however, every time it voluntary crossed to the dark box (CS), it received an electric foot shock (US) that the rat fears (UR). This punishment reinforced the avoidance of the dark box (CR), tested by withholding responses of the rat. C) Social fear conditioning, voluntary social approach (CS) is punished like in B, which promotes social avoidance (CR, see *section 1.4.3*). Image created with BioRender.com.

Passive avoidance and its relevance in modelling psychiatric disorders

Estes and Skinner (1941) adapted the Pavlovian principles to conditioning of voluntary behaviors, known as *operant or instrumental conditioning* (Estes and Skinner, 1941; Fanselow and Ponnusamy, 2008). Here, I focus on the *passive or inhibitory avoidance conditioning* due to its relevance to model psychiatric disorders. Pathological avoidance of specific situations is a recurrent symptom of patients with psychiatric disorders (LeDoux et al., 2017; Nutt et al., 2008). For example, patients with Post-Traumatic Stress Disorder (PTSD, see section 1.2.2 below) avoid environments related to intrusive memories, while social anxiety disorder patients (SAD, see section 1.2.1) avoid social situations, and patients with obsessive compulsive disorder eluded situations that potentially trigger compulsive behaviors (Nutt et al., 2008; Rachman, 2004).

Passive avoidance conditioning induces the suppression of an innate preference or a voluntary behavior to prevent a punishment, which commonly is an electric foot shock (US) (Ogren, 1985). As a result the subject withholding it´s responses (CR)(LeDoux et al., 2017). For example, the

step-through task (Fig. 1.B) punishes the innate preference of the rodents for dark compartments inside the conditioning chamber by an inescapable shock (Ogren et al., 2008). Thereby, the passive avoidance task combines Pavlovian contextual fear conditioning elements with the expression of an instrumental response (LeDoux et al., 2017; Ogren, 1985).

To model SAD a promising mouse model was recently developed in our laboratory, the SFC (Fig.1.C) (Toth et al., 2012b), explained in much more detail in *section 1.4.3*. The SFC is based on passive avoidance conditioning and the first part of the present thesis is devoted to its establishment in rats. Since the SFC model is relatively novel, the theory behind the involved memory processes is still in progress (Masis-Calvo et al., 2018; Menon et al., 2018; Zoicas et al., 2014). However, here, I attempt to conceptually frame the social fear memory result of social fear conditioning.

Social fear memory consists of two components or memory systems, one is the information related to the fear conditioning, the *emotional memory* (the CR, explained in the paradigms above) and the second is the *social recognition, i.e*., the memory of conspecific to whom the adverse event occurred (see below a more detailed definition). This memory dissociation is recognized as well in humans, as emotional and episodic memories. Patient with lesions either in the amygdala or hippocampus show that these two memory systems were separately processed in each region (reviewed in (Fanselow and Ponnusamy, 2008).

Social recognition is the ability to discriminate between conspecifics (Camats Perna and Engelmann, 2017), is a crucial ability that intrinsically regulates social interactions (Holmes and Mateo, 2007; Mateo, 2004). One of the most famous examples of conspecific recognition is the *imprinting* described by the Nobel laureate Konrad Lorenz (Goth and Hauber, 2004; Insel and Fernald, 2004). This specific memory is long-lasting due to the relevance for the offspring of recognized parental figures who provide care and later to avoid incest in some species (see an excellent review in (Insel and Fernald, 2004). The other way around, the parent's ability to recognize their offspring is highly variable and seems to be of more relevance in precocial than altricial species (Goth and Hauber, 2004; Scheiber et al., 2017). Sibling recognition is also well studied (Clemens et al., 2020; Holmes and Mateo, 2007; Porter, 1988). So far, these examples belong to the category of kinship recognition relevant in the light of Hamilton theory (1964) about the nepotism fitness strategy (Hamilton, 1964; Holmes and Mateo, 2007).

In contrast, conspecific recognition of adults (kin independent) seems to be a short-term process, at least in rodents with few exceptions (Camats Perna and Engelmann, 2017; Insel and Fernald, 2004), such as monogamous species like prairie voles, for review see (Walum and Young,

2018). The strength of these memories can be influenced by species, kin, gender, and individual differences (Green et al., 2015; Insel and Fernald, 2004; Scheiber et al., 2017). For an extensive review in rodents see (Camats Perna and Engelmann, 2017; Holmes and Mateo, 2007; Porter, 1988). In rodents, due to their dominant olfactory sense (Johnston, 2003), the innate preference to investigate a novel over a familiar conspecific is a common indicator of successful discrimination (Camats Perna and Engelmann, 2017).

To measure social recognition in rodents the general settings include a *sampling session* (*i.e.,* acquisition of a conspecific memory), where a neutral stimulus can be explored by the experimental subject. This process is influenced by the kind of odor signature (volatile *vs* non-volatile odors), as well the perception of the stimulus (*i.e*., when occurs in terms of developmental windows and for how long?)(reviewed in (Holmes and Mateo, 2007; Johnston, 2003). The time between the sampling session and the memory test is called **consolidation** period or **retrieval interval** (the use of one term over the other depends on referring to either the memory process or the procedural step). Finally, the memory recall takes place where the exploration of a familiar stimulus (*i.e*., stimulus used in the sampling session) is compared to a *novel* stimulus. The stimuli presentation varies between protocols, either simultaneously (called **social discrimination**, see (Engelmann et al., 1995)), serial or a habituation-dishabituation arranges (see a review in (Camats Perna and Engelmann, 2017).

1.1.3 Neurocircuitries underlying fear and anxiety-related behaviors

Neurocircuits related to the control of fear and anxiety-related behaviors vary according to the kind of threat (*e.g.*, predator, aggressive conspecific or pain stimuli) and learning process (Gross and Canteras, 2012; LeDoux and Daw, 2018). The brain regions encoding the sensorial information related to the threatening stimulus would depend on the modal nature of the stimulus. For instance, auditory information is processed by thalamic areas and the auditory cortex, visual cues recruit the perirhinal cortex, and olfactory signatures dependent on the vomeronasal organ and accessory olfactory bulb (AOB) (reviewed in (Fanselow and Ponnusamy, 2008; Insel and Fernald, 2004). Information related to the threatening context involves hippocampal regions (Steimer, 2002). Several brain regions are engaged in the integration of those inputs and the control of the output (*i.e*., fear and anxiety-related responses), such as the amygdala (AMY), hippocampus (HP), lateral septum (LS), prefrontal cortex (PFC), bed nucleus of the stria terminalis (BNST), periaqueductal gray (PAG), and the hypothalamus, especially the paraventricular nucleus (PVN, see *section 1.3.1*).

Uncovering the brain network of fear and anxiety-like behaviors can be challenging (LeDoux and Pine, 2016), for example, the circuits proposed by (Steimer, 2002) in Fig. 2 involved an extensive network. In the following, I will focus only on the function of those areas related to my project.

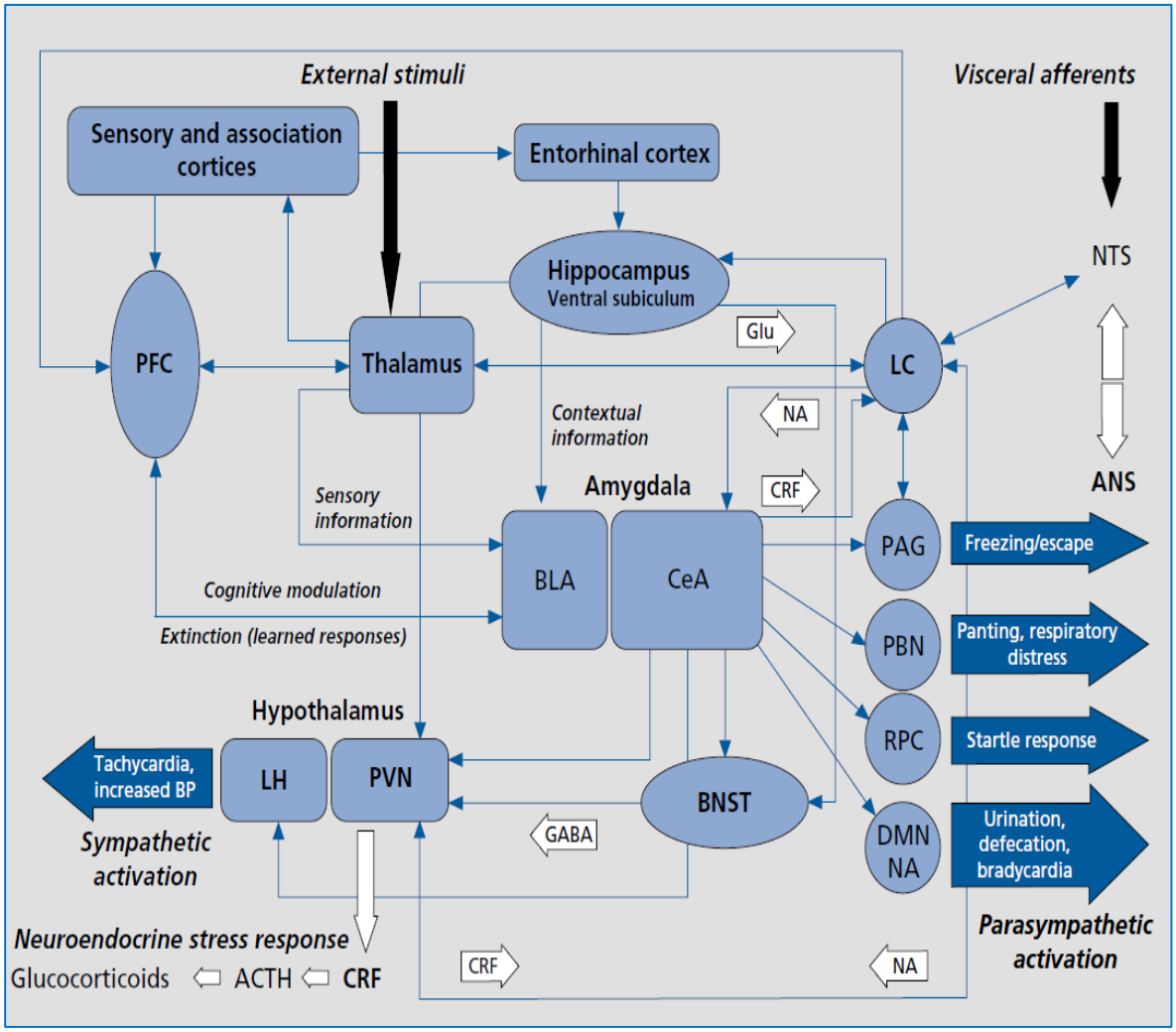


Fig.2. A schematic view of major brain circuits involved in fear and anxiety adapted from (Steimer, 2002). Sensory stimuli are relayed by the thalamus to the amygdala and cortex. The basolateral complex (BLA) of the amygdala is the input side of the system, which also receives contextual information from the hippocampal formation. After intra-amygdala processing of the emotional stimuli, the central nucleus of the amygdala (CEA), sent outputs that activates the locus coeruleus (LC), the paraventricular nucleus (PVN), the lateral hypothalamus (LHP) and the bed nucleus of the stria terminalis (BNST). The latter associated to the modulation of anxiety-related responses. In addition, the CEA directly activates periaqueductal gray (PAG, for freezing or escape), parabrachial nucleus (PBN, respiratory rate), caudal reticulopontine nucleus (RPC, startle), and the dorsal motor nucleus of the vagus (DMN). The prefrontal cortex (PFC) modulates the amygdala responses, and it is also involved in the extinction of fear- and anxiety-related conditional responses. ACTH, adrenocorticotropic hormone; ANS, autonomous nervous system; BP, blood pressure; GABA, γ-aminobutyric acid; Glu, glutamate; NA, noradrenaline; NTS, nucleus tractus solitarius.

Amygdala (AMY)

Our understanding of the AMY function has been refined over the years, currently viewed as a center for gathering the information of emotional stimuli, sorting its valence, and control the appropriate response (Cain and LeDoux, 2008; Gross and Canteras, 2012; Kim et al., 2017). Furthermore, the AMY seems to orchestrate large-scale networks related to different memory processes for emotional experiences (see a review in (Hermans et al., 2014)). Subregions of the AMY react to threatening stimuli, but also respond to appetitive stimuli (reviewed in (Gross and Canteras, 2012).

Anatomically, it can be broadly divided into the basolateral (BLA), medial (MEA), and central amygdala (CEA) (Gross and Canteras, 2012). BLA is involved in associative learning between sensory cues and conditioned responses. The BLA regulates the activation of CEA that controls fear responses (Fig.3, see more details below) (Gross and Canteras, 2012). Besides, the BLA is involved in extinction learning and receives inputs from areas such as the PFC and the HP (Steimer, 2002). When a threat is uncertain, connections from the BLA and HP lead to engagement of the BNST in the control of anxiety-related responses (LeDoux and Pine, 2016).

Notably, the MEA (not shown in Fig.2) reacts to a predator or conspecifics odors and its specific dorsoventral activation pattern seems to identify the relevance of these stimuli (Lukas et al., 2013; Samuelsen and Meredith, 2009). Arakawa and colleagues show that the MEA regulates the approach/avoidance of healthy or sick conspecifics in rats, respectively (Arakawa et al., 2010). The CEA has afferences to stress-related regions such as the locus coeruleus (LC), the PVN, the lateral hypothalamus (LHP), and the BNST (Steimer, 2002). In addition, the CEA control fear responses, it directly activates the PAG (*i.e.*, freezing or escape), parabrachial nucleus (PBN, respiratory rate), caudal reticulopontine nucleus (RPC, startle), and the dorsal motor nucleus of the vagus (DMN). Lesions of the CEA block these fear responses (reviewed in (Roelofs, 2017).

Hippocampus (HP)

The HP processes diverse information, such as spatial information (where?), temporal information (when?), the kind of event (what?), and social recognition (who?) (Okuyama, 2018). The HP is also referred to as Ammon's horn or ***cornu ammonis*** **(CA)** and it´s anatomically subdivisions are the CA1, the CA2, and the CA3. The dentate gyrus (DG) is an additional area of the HP, which integrates the information from entorhinal cortex (Amaral et al., 2007; Rebola et al., 2017). The DG projects to the CA3, that is essential for the rapid encoding of memory (Kesner, 2007; Rebola et al., 2017).

Moreover, the CA3 and CA1 are both related in the acquisition of context-dependent extinction, however, only CA1 is engaged in the retrieval of this kind of memory (Rebola et al., 2017). Current studies suggest that dorsal CA1 is also involved in the "self" and "other conspecifics" spatial representation in rats and bats (Danjo, 2020; Omer et al., 2018). Mice studies show that the CA2 is essential for social recognition and aggression, mediated by the vasopressin 1b receptor (V1b) (Pagani et al., 2015; Smith et al., 2016). Furthermore, the HP can be also divided along the septotemporal axis tie to different functions; the dorsal region is involved in spatial learning and memory (Moser and Moser, 1998), whereas the ventral region plays a role in modulating anxiety-related behaviors in an anxiogenic manner (Bannerman et al., 2004; Bertoglio et al., 2006).

Locus coeruleus (LC)

Studies in rats, cats, and monkeys report that increased activation of the LC is associated with alertness and selective attention during acute exposure to threatening stimuli (Breton-Provencher and Sur, 2019; Steimer, 2002). The LC contains a large proportion of noradrenaline (NA) neurons part of the autonomic response mentioned before and is especially sensitive to social stress (Zitnik et al., 2016). The LC project to the PVN and activate the HPA axis (*see section* 1.3.1), it is also connected to other relevant regions, such as the CEA, PAG, PFC, and BNST (Fig.3). Importantly, the LC integrates external but also internal visceral stimuli for the nucleus tractus solitarius (NTS), which highlights its function in response to stress and fear/anxiety situations (Steimer, 2002).

Lateral septum (LS)

The LS is a critical converging point of information from several brain regions (Sheehan et al., 2004). For instance, the LS receives inputs from the PFC, HP, and LC, and it maintains reciprocal communication with areas such as hypothalamus, thalamus, AMY, BNST, PAG, ventral tegmental area and sends unidirectional projections to the nucleus accumbens (NAc) (reviewed in (Deng et al., 2019; Sheehan et al., 2004)). This diverse connectivity may explain the control of equally diverse responses, such as the stress response (inhibition of HPA axis activity), fear (especially conditioned social fear), conspecific aggression, social recognition (kin and non-kin recognition, see also *section 1.3.3*) and other memory processes (Aleyasin et al., 2018; Clemens et al., 2020; Koolhaas et al., 1999; Menon et al., 2018; Niewiadomska et al., 2009; Thomas et al., 2013; Zoicas et al., 2014).

1.2 **Fear and anxiety-related psychiatric disorders**

Fear and anxiety are protective behaviors in humans (Gilbert, 2001; Rapee and Spence, 2004). Traditionally, a pathological condition is considered when excessive fear or anxious responses (either intensity or duration) are displayed. However, individual differences in those behaviors are often distributed gradually, and the opposite extreme response is equally detrimental (Neumann et al., 2011). For example, considering the negative consequences of a fearless response, such as the Urbach-Wiethe disease, which patients suffer an impaired recognition of negative emotional expressions and impaired contextual learning of fear due to symmetrical damage in the AMY, or the Williams syndrome, characterize by overly friendly to strangers but difficulty to establish relationships (Järvinen et al., 2013; Siebert et al., 2003). In general, inappropriate fear and anxiety responses lead to various pathologic conditions, for review see (Buckley et al., 2009; Buske-Kirschbaum et al., 2001; Cohen et al., 2007; Reiche et al., 2004; Singewald et al., 2015).

Pathological states of these behaviors are classified mainly by psychological criteria, *i.e*., with tools such as the Diagnostic and Statistical Manual for Mental Disorders (DSM) and International Classification of Diseases. Nevertheless, the neurobiological underpinnings are still poorly understood (Leichsenring and Leweke, 2017; Nestler and Hyman, 2010). The need to improve our knowledge in this regard is also highlighted by the pandemic proportions of those psychiatric disorders, as well as the reduced efficacy of the current treatments (Neumann and Slattery, 2016; Singewald et al., 2015). It is a problem that should be face by top-down policy health makers, considering the economic burden related to psychiatric disorders. In the European union its related costs were estimated to around 74.4 billion Euro (Gustavsson et al., 2011). In the following sections, I focus on two of the psychiatric disorders, SAD in the light of the SFC model (results part I) and the PTSD in the case of the CFC (results part II).

1.2.1 Social Anxiety disorder (SAD)

SAD is defined by a persistent and exacerbated social fear and avoidance of social situations following the criteria of the DSM-V (APA, 2013). Patients who suffer SAD deal with a constant negative cognitive bias (*e.g*, fear of being judged by others, strong self-insecurity), which in turn maintains the avoidance behaviors (Nutt et al., 2008). SAD patients also show intense physiological stress responses in social situations*, e.g.* increased heart rate, blushing, sweating, and trembling (Elzinga et al., 2010; Stemberger et al., 1995). Moreover, functional magnetic resonance imaging (fMRI) studies reveal that SAD patients show a greater activation of the AMY in response to harsh

facial expressions compared to healthy controls (Goldin et al., 2009; Labuschagne et al., 2010; Minkova et al., 2017). Similarly, fMRI data on emotional face perception found a lower hippocampal activation in SAD patients compared to panic disorder and healthy control groups, authors suggest that this feature may be used as a biomarker for SAD (Pantazatos et al., 2014). Unfortunately, for SAD patients dealing with common social situations results in a considerable challenge since earlier age (circa 13 years old) (Kashdan and Herbert, 2001; Kessler et al., 2007; Sumter et al., 2009). Furthermore, its lifetime prevalence is about 10.7 % in the USA, with a higher prevalence in females (Kessler et al., 2012).

SAD patients can be treated by psychotherapy, which includes exposure therapy (patients are gradually exposed to social stimuli), cognitive restructuring (identifying anxiety before occurring and apply relaxation techniques), and social skills training (Fedoroff and Taylor, 2001). Among those, the cognitive-behavioral therapy (CBT) is considered as the first-line option (Blanco et al., 2013; Leichsenring and Leweke, 2017). Pharmacotherapy for SAD is also available, for example, selective serotonin reuptake inhibitors (*e.g.* fluoxetine) are considered as the gold standard medication in the short-term (Leichsenring and Leweke, 2017; Stein and Andrews, 2015). Other options are benzodiazepines (*e.g.* clonazepam), monoamine oxidase inhibitors (*e.g*. phenelzine), and ß-blockers (*e.g*. propanolol) in cases of SAD due to performance (Fedoroff and Taylor, 2001). An extensive review of the pharmacological targets in clinical assays for SAD can be found in (Singewald et al., 2015). Despite these treatment options, SAD patients achieve only partial remission and have a high rate of relapse (Leichsenring and Leweke, 2017; Stein and Andrews, 2015).

Furthermore, SAD is associated with a high socioeconomic burden, 90% of SAD patients report psychosocial impairments (*e.g*., increased risk of dropping out of school, reduced workplace productivity, and reduced socioeconomic status), and more than one-third report severe impairments (Leichsenring and Leweke, 2017). Importantly, a majority of SAD patients report comorbidity with at least one other psychiatric disorder, such as agoraphobia, depression, or substance abuse (reviewed in (Leichsenring and Leweke, 2017; Nutt et al., 2008)).

Altogether, this is highlighting the need for a better understanding of the etiology of SAD, that not only lead to finding more effective treatments but also reducing the risk of other comorbidities and the associated economic cost (Neumann and Slattery, 2016). From the preclinical research point, this is demanding to improve the current animal models for SAD. One approach to achieve this is include developmental risk factors in the research design (Nestler and Hyman, 2010). In this regard, SAD is associated with environmental factors, including parental influences (Ollendick and

Hirshfeld-Becker, 2002). Parents who suffering from SAD avoid social interactions and thereby influence their children's social relationships (Daniels and Plomin, 1985). From the genetic perspective, studies showed a heritability of around 15-50 % (Kendler et al., 1999).

Moreover, there is evidence that **conditioning events** and **behavioral inhibition trait (BI)** to unfamiliar situations are also risk factors (Ollendick and Hirshfeld-Becker, 2002). Therefore, I aimed to include these factors to model SAD in rats in the first part of the present thesis (see sections 1.4 and 1.5). Regarding the conditioning events, several studies found that *circa* 44-66 % of SAD patients recall a traumatic social event that matches with the onset of SAD symptoms (Mulkens and Bögels, 1999; Öst and Hugdahl, 1981; Ost, 1985; Stemberger et al., 1995). BI is a construct for vulnerability to anxiety disorders, it implies an increased sensibility of the individuals to threats (*i.e.*, social evaluation and social trauma). BI during early life proves to be a strong predictor of SAD. For instance 72% of SAD patients report this trait during their childhood (Stemberger et al., 1995). Moreover, BI was the single greatest predictor to develop SAD (nearly 50%) in a meta-analysis of highly behaviorally inhibited children (Clauss and Blackford, 2012). For an extensive review of BI that includes its interactions with other risks factor see (Spence and Rapee, 2016).

1.2.2 Post-traumatic stress disorder (PTSD)

PTSD was recently reclassified in a new category as trauma- and stressor-related disorder in DSM-V (APA, 2013). PTSD is developed in a few subset of individuals (about 10-20%) after a traumatic experience (*i.e.*, exposure to actual or threatened death, serious injury, or sexual violence) (Fanselow and Ponnusamy, 2008). Part of the pathology is re-experiencing the traumatic experiences triggered by a similar stimulus or less intense stressors (Rosen and Schulkin, 1998). This indicates an impaired ability in fear extinction learning (Yehuda and LeDoux, 2007). Other symptoms are high avoidance, nightmares, and dysregulation in mood, cognition, and the HPA axis (APA, 2013). Compared to trauma-exposed controls the patients with PTSD respond to emotional stimuli with a decreased activation in medial PFC and an opposite response in the AMY and HP, reviewed in (Zoellner et al., 2020). PTSD prevalence range from 6 to 7% (Kelmendi et al., 2016). However, studies with specific sample sets such as Vietnam war veterans or female rape victims have reported a lifetime prevalence as high as 30% (Andrews et al., 2003).

First-line treatments of PTSD are varieties of CBT, yet only 50% of patients show remission (Richter-Levin et al., 2019). Common pharmacotherapy includes selective serotonin reuptake inhibitors (*e.g.* paroxetine and sertraline) that show mild-to-moderate success (Kelmendi et al.,

2016). Moreover, PTSD has a high level of comorbidity with other psychiatric disorders, such as GAD, specific phobias, depression and substance abuse (Nutt et al., 2008). Notably, PTSD presents a strong sex bias (*i.e*., women are twice as likely to develop it than in men). Nevertheless, sex-specific markers related to this susceptibility remain poorly explored in the preclinical research (Shansky, 2015). For instance, overall extinction literature only less than 2% of the evidence refers to females (Lebron-Milad and Milad, 2012). The specific factors that putatively underlie this sex bias will be described in section 1.3.2. Together, the weak response to treatments (*i.e*., psycho- or pharmacotherapy), and the sex bias observed in PTSD population, highlight the need not only to carry research in new treatment targets but also to include females in the preclinical research. Regarding this, the second part of my thesis focused on the effects of NPS (a novel target, see *section 1.3.3)* in a model of PTSD (*section 1.4.4*) in female rats.

1.3 **Factors of susceptibility to psychiatric disorders**

Regulation of fear and anxiety and its pathological states remain poorly understood (Steimer, 2002; Yehuda and LeDoux, 2007). Many factors have been targeted for research such as abnormal stress response, genetic components, sex differences, and environmental factors, among others (Davidson and McEwen, 2012; Galea et al., 2020; McEwen et al., 2015; Pinares-Garcia et al., 2018; Singewald and Holmes, 2019; Steimer, 2002; Tost et al., 2015). However, it is currently accepted that more than one single factor or a combination of them which fine-tune the individual's response. Here, I review some aspects that were part of my research projects, such as stress, the female specific-risk and the putative disbalance in some neuropeptide systems.

1.3.1 Imbalance of the stress response

Activation of the stress response is tightly linked to fear and anxiety-related behaviors (Apfelbach et al., 2005). Indeed, there is a potential overlapping that made it difficult to separate the causal links in this relationship. On one hand, anxiety and fear can be a part of the stress response, on the other hand, anxiety and fear constitute potential stressors (Blanchard and Blanchard, 2008). The task to understand this relationship become even more complex if we consider the broad aspects regulated by the stress neurobiology (*e.g*., endocrine, immune, cognitive, motivational, and behavioral)(Frank et al., 2016; Sapolsky, 2015).

Therefore, I am interested here on the stress influence on the memory process related to threatening situations. This is considered a key point to understand some of the fear-and anxiety-

related disorders in humans (Yehuda and LeDoux, 2007). For instance, as an etiology factor of PTSD (*i.e*. vulnerability to acquire endure fear memories resistant to extinction), as well as potential factor to improve the psychotherapy interventions (*i.e.,* modifying established fear memories or learning safe cues respect to social interactions in case of PTSD and SAD, respectively). Hence, in this section, I will describe the stress response and later some aspects of its influence on the memory process.

Stress response is defined as an interaction between stimuli (stressors) and the homeostatic systems, such as the SNS and the HPA axis (Herman et al., 2016). The SNS (Fig. 3) is the fastest to react to a stressor and consists of preganglionic neurons originating in the thoracic and lumbar regions of the spinal cord that connect to postganglionic neurons (Ulrich-Lai and Herman, 2009). The latter innervate target organs via the neurotransmitter's noradrenaline and adrenaline, whereby adrenaline occupies a major part in maintaining the internal constancy (Steimer, 2002).

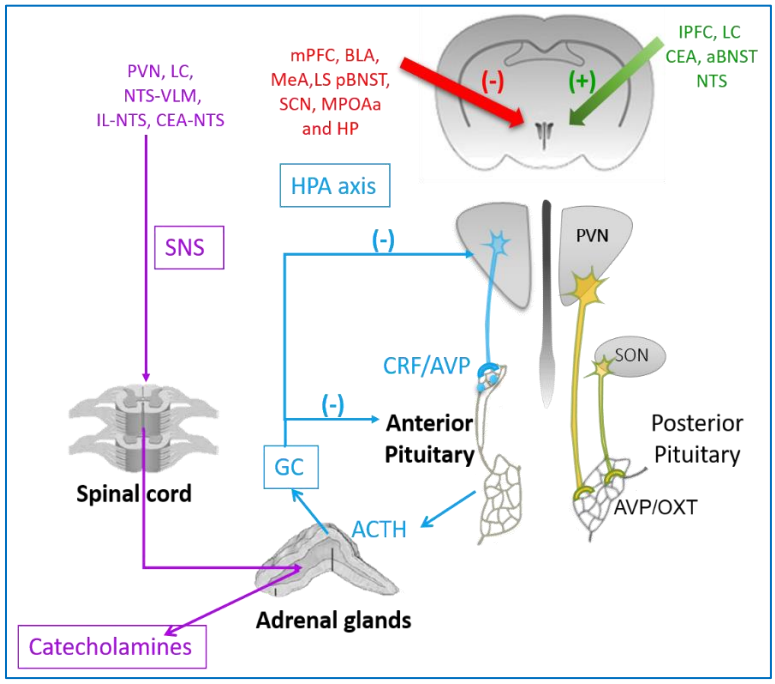


Fig.3. Stress response systems a simplified scheme based in (Ulrich-Lai and Herman, 2009).
Sympathetic nervous system (SNS, in purple): preganglionic neurons receive inputs from regions such brainstem, paraventricular nuclei (PVN), Locus coeruleus (LC), nucleus tractus solitarius (NTS, that integrates signals from infralimbic cortex IL-NTS or central amygdala CEA-NTS). Then postganglionic neurons target organ´s response, such adrenal catecholamines release.
Hypothalamic-pituitary-adrenal (HPA, in blue) axis: parvocelullar neurons (blue color) release corticotropin releasing factor (CRF) and vasopressin (AVP), which causes the anterior pituitary to secrete adrenocorticotropic hormone (ACTH), which in turn is transported by the circulation to the adrenal glands, stimulating the synthesis and release of glucocorticoids (GC). Excitatory (green arrow) and inhibitory (red arrow) inputs to PVN. Abbreviations: medial prefrontal cortex (PFC), medial amygdala (MEA), basolateral amygdala (BLA) and hippocampus (HP), Lateral septum (LS), medial preoptic area (mPOA), suprachiasmatic nucleus (SCN), supraoptic nuclei (SON), anterior (aBNST) and posterior (pBNST)areas of bed nucleus of the stria terminalis. Nucleus of the solitary tract (NTS). OXT: oxytocin. (-) Negative feedback loops.

Stress in general results in the activation of the HPA axis (Fig.3), starting with the corticotropin releasing factor (CRF) neurons in PVN. CRF is released and reaches the anterior pituitary via portal blood capillaries of the pituitary stalk, then it binds to CRF receptors type 1 (CRFR1) (Herman et al., 2016). Activation of CRHR1 and subsequent intracellular signaling cascades involving cAMP that the adrenocorticotropic hormone (ACTH) release to the periphery (Neumann et al., 1998a). ACTH then binds to melanocortin-2 receptors in the adrenal cortex, and its activates a synthesis pathways, promotes the release of glucocorticoids (GC). The GC, cortisol in humans and corticosterone (Cort) in rodents, ultimately coordinate the many peripheral effects, and the recruitment of energy resources, and the immune system (Lupien et al., 2007). There is several points of negative feedback on hippocampal, hypothalamic and pituitary structures that shut down the HPA axis to return to basal homeostasis (for review see Herman et al., 2016).

Cort actions is regulated by different features, such binding to the corticosterone-binding globulin, only free GC cross the blood-brain barrier or cell membranes (circa 10% of GC is unbound) (Lupien et al., 2007). Besides, Cort release follows a circadian rhythm (low levels in morning *vs* high levels in the evening)(Reul et al., 1987). After release, Cort binds manly 2 types of receptors: mineralocorticoid receptor (MR or type 1) and glucocorticoid receptor (GR or type 2). The former has a 10-fold higher affinity to bind Cort than the second (Kloet et al., 2005). Thus, MR receptors may be engaged under basal Cort levels, while, GR receptors are involved during stress situations (Roozendaall et al., 1996). MR receptors are distributed predominately in HP, the septum and CEA (Joëls and de Kloet, 1994). GR receptors are present as well in those limbic areas but in addition are also in the cerebral cortex and the NTS (Cordero and Sandi, 1998; Davies and MacKenzie, 2003).

GC in general affect emotional responses and cognitive processes (de Quervain et al., 2017b; Sapolsky et al., 2000). In this regard, MR activation have been associated with the response to stressful experiences, whereas GR activation to memory consolidation (de Quervain et al., 2017a). GC either enhancing or impairing memory processes depending on concentration and timing of administration. For instance, GC seem to have a crucial function in memory consolidation of emotional experiences, which involves the BLA and HP function (McGaugh et al., 2002; Roozendaal, 2000). PTSD and SAD studies showed that GC enhance the fear memory extinction (reviewed in (Singewald et al., 2015). In contrast, blocking the Cort availability before the training extinction can attenuate the memory formation for a variety of stressful learning tasks (de Quervain et al., 2017a). A common blocker of the Cort synthesis, approved by the FDA is the metyrapone that selective inhibit the activity of CYP11B1 and CYP11B2 (Fleseriu and Castinetti, 2016). In part of the

experiments here, I test the hypothesis of Cort as enhancer of social fear consolidation memory in rats (Part I).

1.3.2 Female-specific risk in psychiatric disorders

Sex differences have been recognized as a significant factor for the development of psychiatric disorders with women being distinctly more affected than men, for example, in depression or social anxiety (Gogos et al., 2019; Gurvich et al., 2018; Kessler, 2003; Pinares-Garcia et al., 2018). Despite better recognition of the significance of sex-specific research, progress has been slow regarding the bias of studies performed only in males (Heidari et al., 2016; Liu and Dipietro Mager, 2016; Shansky, 2015). Even more relevant is that treatment guidelines are based largely on men data (Liu and Dipietro Mager, 2016; Wizeman, 2012). Consequently, this negatively impact our knowledge of the development, symptomatology, as well effective treatments for various pathophysiology conditions in women (Clayton and Collins, 2014; Coen and Bannister, 2012).Altogether, these strongly emphasize the need for sex-specific research in pathophysiology studies to improve the healthcare of women population (Becker et al., 2005; McCarty, 2016).

In this regard, a starting point in the preclinical research is to study the contribution of the sex hormones, as one of the main factors that differ between male and females, for a review see (Pinares-Garcia et al., 2018). Sources for sex hormones variation includes puberty, estrous cycle, pregnancy and lactating period and menopause (Gogos et al., 2019; Pinares-Garcia et al., 2018; Zuloaga et al., 2020). This innate female variability should be embraced it instead of considering it as a reason to neglect the research in females. Therefore, it is important to concentrate the research regarding stress-related and fear-anxiety-related disorders on females in different reproductive states. In this regard, the second part of my thesis is focus on extend our knowledge of the Neuropeptide S effects (*see section 1.3.3*). In specific, its contribution to fear and stress response virgins and lactating female rats. Hormones in virgin adult females naturally cycling, thus, I take smears to control the putative effects of estrous cycle (see an overview below), and lactating females showed a particular hormonal profile that carry a broad spectrum of adaptations (see a brief summary below).

Estrous variation

The estrous cycle lasts for 4-5 days in adult female rats; it consists of four phases: diestrus, proestrus, estrus, and metestrus; it is regulated by hormones, such as luteinizing hormone (LH), follicle-

stimulating hormone (FSH), progesterone and estrogen. In the following, I briefly summarized the hormonal changes associated to each phase (reviewed in (Westwood, 2008): Progesterone levels increases during diestrus phase, followed by a rise in estrogen levels during proestrus phase causing the peak of LH and FSH. Then, ovulation occurs, and the female become sexually receptive during estrous phase. In the absence of conception, the progesterone levels decrease, and the cycle continue into metaestrus phase mainly marked by FSH levels. These hormone fluctuations influence the HPA axis activity; thus, the estrus cycle may play a major role in stress-related affective diseases (Figueiredo et al., 2002; Zuloaga et al., 2020). Cort levels reach the highest levels in proestrus, whereas the lowest levels are observed during the estrous phase, without alter the ACTH levels (Atkinson and Waddell, 1997). Regarding sex differences, during the circadian rhythm, virgin rats showed higher pulses of Cort compared to males (Figueiredo et al., 2002; Seale et al., 2004). Similarly, during stressful events, females release higher levels of ACTH and Cort than males (Figueiredo et al., 2002; Mevel et al., 1979)

Lactating period

This period is characterized by amazing neuroplasticity, were several physiological, behavioral, and cognitive adaptations contribute to the well-being of both mother and its offspring (Bosch and Neumann, 2012; Hillerer et al., 2012; Neumann et al., 1998a; Slattery and Neumann, 2008; Stolzenberg et al., 2019). Adaptations including induction of maternal behavior, increased of food and water intake, termination of reproductive cyclicity, and variations in the HPA axis (Russell et al., 2001; Slattery and Neumann, 2008; Smith et al., 2006). The brain regions AMY and PVN, involved in the regulation of emotions and of the HPA axis, belong to complex neural networks that are important mediating maternal behavior in lactating rats (Bosch and Neumann, 2012; Brunton and Russell, 2008). In this regard, the basal activity of the HPA axis is altered during late pregnancy and the lactating period, where increased plasma Cort levels were observed (Neumann et al., 1998a; Neumann et al., 2001). In contrast, there is a hypo-responsiveness of the HPA axis to stress reflected by lower ACTH and Cort levels, accompanied by reduced CRF and AVP mRNA expression in the PVN (reviewed in (Brunton and Russell, 2008; Stolzenberg et al., 2019). This differential HPA axis response, either pre- and postnatal may protect the offspring and serve the dams as well to properly care of the pups (Slattery and Neumann, 2008). Nevertheless, important to mention that this period is also related to an increased risk for cardiovascular, metabolic, and neuropsychiatric diseases (Brummelte and Galea, 2016; Galea et al., 2020). For the latter, the interaction between

the HPA axis and the sex hormones is considered part of the etiology, however, is still poorly understood (Stewart and Vigod, 2019).

1.3.3 Neuropeptides systems that regulate fear and anxiety responses

The fear and anxiety responses not only affect the behavioral readout, are often accompanied by a variety of autonomic responses (*i.e*., cardiovascular, gastrointestinal, vegetative, among others), and present a tight link with stress systems, such as the HPA axis (Steckler, 2008). Is from this perspective that many neuropeptide systems have emerge as promised treatment for psychiatric disorders, due to its broad modulation patter, and especially true for those associated to socio-emotional behaviors (Grinevich and Neumann, 2020; Sippel et al., 2017). The later considering that some disorders, such as SAD and PTSD are related with social trauma as risk factor or as a consequence of their symptoms (*i.e*., social avoidance) negatively impact the social interactions of those patients (Nutt et al., 2008). Here, I review the contribution of some neuropeptide that fulfill the above description, such as vasopressin, oxytocin, and the neuropeptide S.

Vasopressin (AVP) and Oxytocin (OXT)

This section was adapted from my own review (Masis-Calvo et al., 2018)

AVP and OXT are nonapeptides that evolved from gene duplication (Caldwell, 2017), which different homologues are broad represent among invertebrate and vertebrate taxa (see a review in (Jurek and Neumann, 2018)). Both are synthesized mainly within magnocellular cells of the SON and PVN, transported via neurohypophysis, and finally released into the blood to fulfill different functions. Indeed, their names were inspired by some of the first describe functions in the periphery , such as water balance and parturition, among other autonomic aspects (Caldwell, 2017). These nonapeptides can also be released within the brain, produced by parvocellular cells, from which projecting to extended brain network (Bosch and Neumann, 2012; Hernández et al., 2016; Jurek and Neumann, 2018). AVP effects depend on three distinct receptor subtypes: V1a, V1b and V2 receptors (Caldwell, 2017). Especially, the V1a receptor has been implicated in social and defensive behaviors. OXT has only one receptor, however, OXT can bind to AVP receptor as well (Manning et al., 2012; Song et al., 2014).

AVP and OXT systems become activated in a stressor-specific way (Engelmann et al., 2004; Neumann, 2007). Magnocellular neurons respond with the secretion of OXT in response to non-social stressors and some social interactions, such as mating and maternal-offspring interacting

during nursing (see review in (Jurek and Neumann, 2018)), but remain largely unchanged to social stressors (Neumann, 2007). Curiously, AVP is not secreted into the blood during exposure to stressors, except those resulting in increased plasma osmolarity, such as exercise (Landgraf et al., 1982; Wotjak et al., 1996).

The intracerebral release of these neuropeptides occurs in a strict brain region- and stimulus-dependent manner. AVP and OXT can be released in a somato-dendritic fashion within the PVN and SON, or by axons projecting to limbic areas, such the central amygdala and hippocampus, which are recognized areas for modulating the stress response (Hernández et al., 2016; Knobloch et al., 2012; Neumann, 2007). For instance, social stress (e.g., social defeat) selectively activates AVP release within the PVN, but not SON (Wotjak et al., 1996), while OXT release was found within the SON, but not PVN(Engelmann et al., 1999). Curiously, the intra-PVN release of AVP can be observed mainly in animals with active coping styles (Ebner et al., 2005). Thus, the context of social interaction is relevant for activating the AVP and OXT systems.

AVP in social recognition and memory consolidation

AVP is crucial in the regulation of social behaviors. In this regard, neuropeptides in the arginine vasotocin/arginine vasopressin family are known to influence social recognition, social communication, and aggression in animals (see a review (Albers, 2015; Caldwell, 2017)). Substantial evidence supports the role of AVP in social recognition. Central administration of AVP improves the social recognition in rats (Le Moal et al., 1987). In the same line, local infusions of AVP in the lateral septum (LS) prolong the time period in which an animal is capable to socially discriminate conspecifics, whereas a selective antagonist (V1aR-A) impair the social recognition (Bielsky and Young, 2004; Dantzer et al., 1988). In the same direction, increase in the vasopressin V1a receptor expression in the LS increased social recognition (Landgraf and Wigger, 2003). AVP in the rat LS is much more abundant in males than in females (De Vries et al., 1981). Taken together, those studies highlight the AVP role in the regulation of social memory abilities, which could be a promising target to treat social traumas.

Neuropeptide S (NPS)

The NPS consists of 20 amino acids, named by its N-terminal serine residue (*e.g.*, the sequence in rats deferring with humans only in four amino acids marked in bold letters: N-SFRNGVG**S**G**V**KKTSF**R**RAK**Q**-C), it is a highly conserved neuropeptide among vertebrates except in

fish (Reinscheid, 2007; Reinscheid and Xu, 2005; Xu et al., 2004). The latter, interestingly suggest that NPS putative regulate terrestrial adaptations, such as sleep-wake cycles (see a review in (Reinscheid, 2007)). *NPS* mRNA is expressed in a few brain areas, such as the hypothalamic nucleus, the AMY, and the LC where is localized its stronger expression (Clark et al., 2011; Xu et al., 2007). In contrats, the distribution of its receptor (NPSR) show a wide expression in the brain, the higher receptor expression are localized in olfactory regions, cortical regions, and limbic regions (Xu *et al.,* 2004, 2007). In the periphery, the NPSR can be expressed in several tissues, however the higher abundance is found in the thyroid, salivary, testis, and mammary glands (Xu et al., 2007). In humans, the NPSR is also ubiquitously distributed for an extensive review see .Its expression pattern may match with the broad effects so far related to NPS/NPSR system (Okamura and Reinscheid, 2007). The NPSR belongs to the G protein-coupled receptors family and its activation coupling of Gq and Gs (Zhang and Tao, 2019). The NPS neurons mediate mainly excitatory signals, for instance, in the brainstem colocalizes with glutamatergic neurons and an small subset with acetylcholine, while in the AMY colocalize with CRF neurons (Jüngling et al., 2012; Xu et al., 2007). NPS fibers from the LC project to the thalamus, hypothalamus, septal, and AMY regions, where it been reported release in respose to stressors (Adori et al., 2016; Ebner et al., 2011).

Preclinical studies revealed a variety of physiological processes where the NPS system is related, including wakefulness (Xu et al., 2004; Zhao et al., 2012), food intake control (Beck et al., 2005; Peng et al., 2010; Ruzza et al., 2010; Smith et al., 2006), antinociceptive effects (Jinushi et al., 2018; Lee et al., 2020). In terms of the stress response, NPS activates the HPA axis by increasing plasma ACTH and plasma Cort (Smith et al., 2014; Smith et al., 2006). Those effects were obseved either after central (icv), intra-PVN and AMY applications. Moreover, *in vitro* studies showed that NPS increase the CRF and AVP release from hypothalamic cultures, suggesting a potential way that mediates the NPS effects on the HPA axis activity (Smith et al., 2006). From the emotional perspective, NPS has been wellknown by its anxiolytic and panicolytic effects (Cohen et al., 2018; Pulga et al., 2012; Wegener et al., 2012; Xu et al., 2004). NPS was also found to reduce intermale aggression (Beiderbeck et al., 2014). Further, has cognitive effects, were NPS facilitates either fear extinction (Jüngling et al., 2008; Sartori et al., 2016; Slattery et al., 2015; Zoicas et al., 2016), facilitates spatial memory (Han et al., 2009; Okamura et al., 2011), and enhance novel object but not social recognition (Lukas and Neumann, 2012). In summary, NPS system regulate some homeostasis processes and relevant behavioral response. However, most of the studies, as a general pitfall in preclinical research, has been done in males with few exceptions (Germer et al., 2019;

Kreutzmann et al., 2020; Wegener et al., 2012). Therefore, is important to fill the gap of information in regard to female respose to NPS, especially for traslational purposes. In this regard, NPSR single-nucleotide polymorphisms (SNPs) were found associated with asthma, high serum immunoglobulin E, rheumatoid arthritis, inflammatory bowel disease, and perhaps the more relevant for mood disorder panick attacks in humans (reviewed in (Zhang and Tao, 2019)).

1.4 **Modeling fear and anxiety in rodents**

1.4.1 Animals models and its validation

Animal models are essential tools that in broad terms allow us to replace interventions in humans to study psychiatric disorders (McNaughton and Zangrossi, 2008), mostly by ethical or pragmatic reasons rather than for simplicity. In this regard, they have an inherent limitation since psychiatric disorders cannot be fully captured by a single animal model (Nestler and Hyman, 2010). Therefore, the validation criteria that each animal model must fulfill depend on its own purpose (Joel, 2006; Neumann et al., 2011). Common criteria used to validate an animal model were proposed by Willner in 1984 (Fig.4), which includes the *face, predictive, and construct validity* (Willner, 1984). However, are not the only criteria to evaluate the animal models, see an extensive review in (Belzung and Lemoine, 2011).

The first criteria refer to the resemblance of the behavioral or physiological response compared to the human condition, a detailed review of the anxiety disorders symptoms and theirs equivalent behaviors in rodents can be found in (Cryan and Holmes, 2005). It is important to bear in mind that more than a superficial similarity what this validity implies is to consider the homology of the studied measure (McNaughton and Zangrossi, 2008). To illustrate this aspect, we can think of the “bare-teeth” gesture in some monkeys that seems to resemble a human smile (Fig.5). However, not only the facial muscles recruited but its meaning (*i.e*. fear or submissive response, also called grimace) greatly varies across primate species according to social hierarchy (Burrows, 2008; Parr and Waller, 2006). As we can appreciate, this will demand a good knowledge of the animal model used (from a phylogenetic, anatomical, and ethological perspective), since the relevant issue is the functional matching of the behaviors across species (Blanchard and Blanchard, 2008).

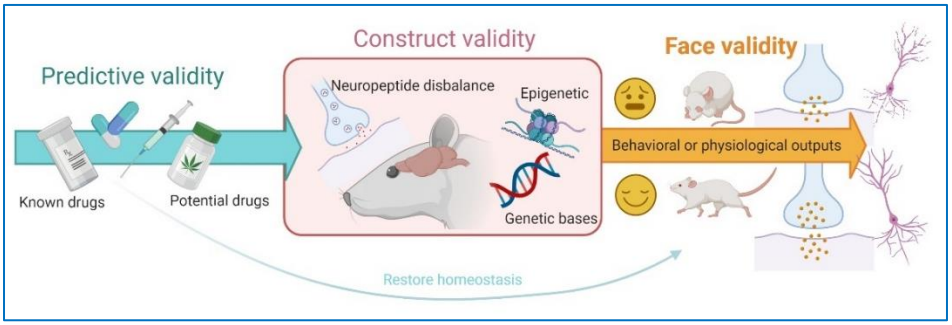


Fig. 4. Types of validity to evaluate an animal model based on Willner´s criteria (Willner, 1984). Image created with BioRender.com.

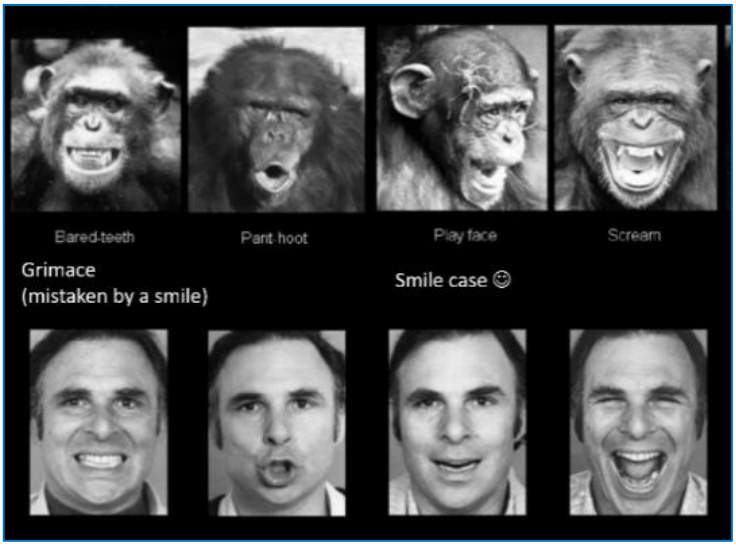


Fig.5 Prototypical chimpanzee facial expressions and homologous facial movements in humans. Ekman pictures adapted in (Parr and Waller, 2006). As can be appreciated in the first picture in the upper side, most of us confused a grimace gesture with a smile, when in most of the primate species is related to an aggressive context or submission gesture. -*Indeed, its homologous gesture in humans in the bottom part seems more like a false smile/awkward gesture*-. The third upper picture refer to the positive situation, such as play contexts and a kind of smile in humans (third bottom picture).

The predictive validity, as its name self-implies, refers to predict an outcome after a manipulation (*e.g*., in case of model anxiety states the positive response of animals to an anxiolytic treatment or therapeutic strategies). Indeed, it has be considered by several authors as a key aspect, although, a negative response to drug treatment does not automatically invalid a model (McNaughton and Zangrossi, 2008). This issue requires a careful evaluation to not leave out new pharmacological targets.

Finally, the construct validity criteria implies causality (etiologic) mechanisms, in this case, the neurobiology pathways related to the fear and anxiety behaviors (Neumann et al., 2011). It is one of the hardest to achieve, especially in disorders with complex pathogenesis. However, if the

knowledge is available, researcher may achieve construct validity by recreating the human etiologic (Nestler and Hyman, 2010). For instance, in case of a disorder with a clear genetic base, several approaches including selective breeding to obtain a phenotype or direct genetic manipulation would be appropriated (Neumann et al., 2011). Another way to improve the construct validity, is expose the animal to a well-known environmental risk factor underlying the disorder (Nestler and Hyman, 2010).

Animal models

As I mentioned in the beginning, I am interested in the regulation of fear and anxiety-related behaviors and its contribution to the neurobiology knowledge of some psychiatric disorders. For that purpose, I had work in two projects that used conditioning models in rats that I will explain in the next sections. In Part I of my thesis, I implement a neurogenetic animal model of social fear by combining two approaches, the social fear conditioning (SFC, *see section 1.4.3*) and the selectively bred for high (HAB) and low (LAB) anxiety-related behaviors (see *section 1.4.2*). In the second part, I mainly focus on the cued fear conditioning (*section 1.4.4*). A brief overview of both projects is given the aims section (*section 1.5*).

1.4.2 Selectively bred rats for high and low anxiety-like behaviors

Family studies revealed that first-degree relatives of SAD patients have a risk of three times higher to suffer from SAD than relatives of control groups (Spence and Rapee, 2016). This suggests an important genetic aspect of the illness. Endophenotypes might facilitate to identify genes related to social fear and social avoidance in rodents. The benefit of using endophenotypes is that not only one gene is selected, but arrays of relevant neurobiological mechanisms and pathways (Landgraf et al., 2007). Animals selectively bred for either high (HAB) or low (LAB) anxiety-related behaviors based on the elevated plus maze test (EPM) (Liebsch et al., 1998). In specific, HAB rats resemble some characteristics of SAD patients. For instance, HAB rats showed a lower exploration time of the open arm of the EPM and this indicates high anxiety (Landgraf and Wigger, 2002; Landgraf et al., 1999; Liebsch et al., 1998). This trait of high anxiety is accompanied by a hyper-responsiveness of the hypothalamic-pituitary-adrenal axis (Landgraf et al., 1999). These characteristics resemble the BI that we discussed above, as an important factor to model SAD. In this regard, an increased cortisol stress-responsiveness significantly correlated with the social avoidance behavior in patients with SAD (Roelofs et al., 2009). Furthermore, there is also a significant correlation between the high

anxiety state in HAB rats and AVP expression (Landgraf et al., 2007). HAB rats showed higher activation of the AVP system due to a SNP in localized in the promotor region (Murgatroyd et al., 2004). Notably, AVP also regulates various social behaviors, especially the social memory (Landgraf et al., 1995). Thus, AVP could be a relevant factor to study the social fear memory in HAB rats.

In contrast, LAB rats showed exact opposite traits compared to HAB rats. For instance, they spent more time in the open arm of the EPM, which is an index of low anxiety response. During maternal separation events, LAB pups showed less ultrasound vocalizations than HAB pups, which also indicated a lower anxiety level (Füchsl et al., 2014). HAB and LAB rats differ in fear learning, where HAB rats showed a delayed extinction (Muigg et al., 2008). Therefore, both lines (HAB/LAB) could give insights into the mechanisms related to vulnerability vs resilience to suffer abnormal social fear.

1.4.3 Social fear conditioning (SFC)

The SFC was developed in mice as an animal model of SAD (Toth and Neumann, 2013; Toth et al., 2012b). As I mentioned before, SFC is a passive avoidance operant conditioning, that induces social avoidance against same-sex conspecifics by punishing direct social contact (i.e., every time they approach the social stimulus, they receive an electric foot shock, 0.7mA). Then, during the extinction phase, three non-social stimuli (empty wire mesh cages) are consecutively presented to prove the fear specificity (e.g., non-social vs. social). Followed by six different social stimuli, subjects initially showed reduced social investigation time, and sometimes freezing behavior, stretched attempts and defensive burying behavior towards the social stimulus (Toth et al., 2013). Typically, repeated exposure of the SFC mice to unknown conspecifics leads to a gradual decline in the fear response. Finally, during the extinction-recall phase (day 3), mice are again exposed to another set of six social stimuli (Toth et al., 2012b).

The SFC in mice promotes a specific induction of social fear without altering other responses, such as general anxiety, depressive-like behavior, or locomotion (Toth et al., 2012b). The social fear memory after SFC in mice can be observed both acutely, *i.e*. after 24 hours, but also up to 15 days after acquisition. Additionally, the induced social fear is sensitive to anxiolytic or antidepressant drugs, such as diazepam or paroxetine (Toth et al., 2012). Furthermore, OXT and NPS systems are associated with the social fear expression (Menon et al., 2018; Zoicas et al., 2016; Zoicas et al., 2014). Recently, a blockage of the metabotropic glutamate receptor subtype 5 and activation of subtype7 proved to impaired social fear extinction (Slattery et al., 2017). So far the only risk-factor

for SAD studied by SFC in mice has been the early life stress (*i.e*., maternal separation), however, it reveal opposite effects to initial hypothesis of vulnerability (Zoicas and Neumann, 2016). The study found that maternal separation facilitates the social fear extinction (Zoicas et al., 2014). Taken together, the SFC in mice resembles not only the main behavioral outcome of SAD (social avoidance), as well the responsiveness to the equivalent treatment strategies (i.e., psycho- and pharmacotherapy), that together fulfill the criteria of face and predictive validity. For a detailed review see (Masis-Calvo et al., 2018).

1.4.4 Cued Fear Conditioning (CFC)

The CFC is a Pavlovian conditioning model (*see section 1.1.2*, Fig. 1 A) that contributed with extensive evidence to our knowledge of the neurobiology of fear response and its memory (Fanselow and Ponnusamy, 2008). CFC consists of three experimental phases: *acquisition* (*i.e*., five CS-US pairings, where the CS is tone and the US an electric foot shock of 0.7 mA. During the first shock the rat vigorously runs, jumps, hops, and vocalizes (Rau et al., 2005). These behaviors would be replaced by freezing (Fanselow, 1982), see explanation in *section 1.1.1*. Next phase is the fear extinction, with rodents repeatedly presented with the CS without the US, which leads to a gradual decrease in the fear responses, similar to the repeated exposure to the feared situation during cognitive-behavioral therapy in humans (Cain and LeDoux, 2008). And finally, the recall phase, where the extinction memory formed the day before is evaluated.

1.5 **Aims of this thesis**

The overall aim of my thesis was to increase our understanding of the regulation of fear and anxiety behaviors by selected neuropeptides, specifically AVP and NPS, as well as steroid hormones (Cort). For that purpose, I aimed to work with two conditioning models in rats, the SFC and CFC paradigms, respectively used to study two important psychiatric disorders, namely SAD and PTSD (see section 1.2). In the first part of my thesis, I gave attention to two risk factors of SAD: the conditioning events (social trauma) and the BI. To mimic these factors, I first established the SFC in rats and then combined it with the use of selective breeding lines, in specific the HAB rats. Their high trait anxiety resembles the BI in SAD patients. Together, I aimed to explore different factors that affect social fear memory consolidation in rats (see section 1.5.1).

In the second part of my thesis, I generally aimed to study the effects of NPS in female rats. The NPS system has been identified as an important neuromodulator involved in fear, anxiety, and stress responses. Due to its potent anxiolytic effects, NPS might represent a treatment option for neuropsychiatric disorders. However, NPS effects have mainly been studied in males and, therefore, little is known about the brain NPS/NPSR system in females. Thus, I aimed to investigate the role of neuropeptide S (NPS) in the fear and stress responses of female rats dependent on their reproductive state (see section 1.5.2).

1.5.1 Study the social fear memory using the SFC in rats

The SFC paradigm has only been established in mice. However, studying rats offers the advantage of displaying more complex social behaviors. Besides, from a methodological point of view, rats allow easy access to brain surgery and blood sampling. Moreover, to study the consequences of a genetic predisposition for either high or low anxiety-related behavior on fear conditioning, I aimed to make use of an existing selective rat breeding line, i.e. HAB rats. Their high trait anxiety, i.e., their characteristic avoidance of unfamiliar situations, resembles the higher avoidance of unfamiliar situations in SAD patients. In summary, I considered an advantage to study the social fear memory in rats. This led to my secondary aims: *i)* to adapt the existing murine protocol of SFC to rats, and *ii)* to compare the three selective breeding lines of HAB, LAB and NAB rats with respect to their SFC and CFC behavior. Moreover, to understand which brain areas contribute to the behavioral differences observed between HAB and NAB rats, I also aimed *iii)* to compare the patterns of neuronal activity (using c-Fos technique) after social fear discrimination phase in two brain areas, the AMY and HP, both related to the processing of emotional memories.

In addition, based on my finding that only HAB rats showed long-term (24 hours) individual recognition, I further aimed to reveal the involvement of AVP and GC, since both are known to participate in the social recognition and memory consolidation process. Previous studies revealed that HAB rats showed an elevated AVP expression and release, and enhanced GC response to stress (see section 1.3.2). In this context, I specifically aimed *iv)* to analyze the effects of AVP and blockade of its V1aR-A with central and lateral septum infusions after social fear acquisition to later evaluate the social fear discrimination. Further, I aimed *v)* to identify the role of GC in the long-term consolidation of social fear memory by several approaches: measuring the Cort release during SFC phases (in HAB and NAB rats), blocking the GC synthesis (in HAB rats) and giving synthetic Cort (in NAB rats).

1.5.2 Evaluation of the role of NPS on fear extinction and stress response in virgin and lactating female rats

The NPS system has been identified as an important neuromodulator of fear, anxiety-related, and stress responses. However, there is little known about how the NPS/NPSR system is regulated in females. Females may response to threatening stimuli with differential sensitivity compared to males, mainly due to hormonal variation. Such variation can respond to the estrous cycling or due to specific reproductive states, such as pregnancy, lactation, or menopause period. Herewith, I will consider the estrous cycle and the lactation period to characterize the response in females. Therefore, I specifically aim to:

i) to compare basal mRNA expression of NPS and its receptor in three brain areas related to the control of fear and anxiety behaviors and the HPA axis response, such as PVN, AMY, and LC.

ii) Based on my finding that regional NPS expression is upregulated in lactating rats, I aimed to test for differential effects of icv NPS on cued fear extinction in the CFC paradigm, as NPS was shown to facilitate fear extinction in males.

iii) As the HPA axis response is severely attenuated in lactating, I aimed to evaluate the effects of icv NPS on Cort release both under basal as well as stress-induced conditions.

Material & methods

2. Materials and methods

2.1 **Animals and housing conditions**

The animal experiments of my thesis were performed on male and female Wistar rats (males: 250-500 g). In part I of the thesis I used rats selectively bred for high (HAB) and low (LAB) anxiety-like behavior, and non-selected (NAB) rats as controls. All animals were bred in the animal facilities of the University of Regensburg. The offspring of HAB and LAB rats were tested on the EPM (see section 2.6.1) at the age of 9-10 weeks to confirm their phenotype. Stimuli rats were obtained from Charles River (Sulzfeld, Germany). Animals were group-housed (60 x 40 x 20 cm) until three days before the SFC or surgery, when they were isolated in observation cages (40 x 36 x 24 cm).

In part II of my thesis, I used female Wistar rats obtained from Charles River (Sulzfeld, Germany). Female rats were split into two groups: virgins that remained group-housed (3-4 animals per cage) and lactating that were mated (*i.e.,* two females were paired with one sexually experienced male). Pregnancy was determined by the presence of sperm in vaginal smears, that day was assigned as pregnancy day 1 (P1) and each male was removed. On P18, both groups were isolated in observation cages. I supplied the dams with paper tissue to build a nest. All behavioral tests were carried out during the light phase between 7:00 am and 5:00 pm. Animals were maintained under standard laboratory conditions (12 h light: dark cycle, lights on at 07.00 h, 22 ±2 °C, 55 ±5 % humidity) with food and water *ad libitum*. The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of Oberpfalz.

2.2 **Surgical procedures**

All surgical procedures were performed under sterile conditions. Animals were anesthetized by isoflurane (Baxter Deutschland GmbH, Germany) inhalation; first, it was delivered placing the animal in a jar for a short period to allow shaving the fur of the surgical area, and later via a facemask device under the control of a precision vaporizer. Deep anesthesia was verified by pinching the toes of the animals; the respiratory rates were monitored throughout the surgical procedure. To avoid hypothermia, each animal was placed on a heating pad. Following surgery, rats received an IP injection of antibiotics (120µl, 10mg/Kg, 2.5%, Baytril, Enrofloxacin; Bayer), analgesic (800 µl, 0.05 mg/Kg, Buprenovet, Buprenorphine, Bayer) and topical application of iodine solution (Betaisodona 10 g/100 ml, Mundipharma GmbH, Germany).

2.2.1 Guide cannula implantation

For icv infusions, guide cannulas (stainless steel, 21 G, 12 mm long) were stereotaxically implanted 2 mm above the right lateral ventricle in the following coordinates: AP: −1.0 mm bregma, ML: +1.6 mm, DV: +2 mm below the surface of the skull (Paxinos and Watson, 1998). The guide cannulas used for local infusions (stainless steel, 23 G, 12 mm long) in the LS were placed at bregma AP: −0.4 mm, ML: +0.7 mm, DV: +2.5 mm below the surface of the skull (Paxinos and Watson, 1998). After an incision in the skin to exposed the skull, two holes were drilled on it and two stainless steel screws were fixed into the skull to provide better adhesion of the dental cement (Kallocryl, Dr. Speier GmbH, Germany) that hold the cannula in place. Finally, the guide cannulas were closed with a dummy cannula (stainless steel, 27 G, 12 mm long, with a bend in the third upper part). To avoid the risk of infection, dummy cannulas were cleaned daily (using 70% ethanol) during the handling procedure.

2.2.2 Jugular vein catheter implantation and blood sampling

Jugular vein catheter implantation

To implant jugular vein catheter (JVC), I followed protocol in (Neumann et al., 1998b). Throughout the surgery the blood flow through the catheter was repeatedly checked. Physiological saline (0.9% NaCl; B, Melsungen, Germany) was used to replace withdrawn blood volume during surgery. The use of Heparin-Natrium25000-ratiopharm (Ratiopharm, Germany) prevented blood clotting in the catheter tubing.

Blood sampling

On the test day, I connected the JVC to a line (consisted of a 35 cm piece of PE-10 plastic tubing) attached to a 1-ml plastic disposable syringe filled with heparin solution. All blood samples (0.3 ml each sample) were collected into EDTA-coated Eppendorf tubes, centrifuged at 5000 rpm for 10 min at 4°C. Plasma aliquots (10 µl) were stored at -20°C until Cort concentrations were assayed by ELISA (see 2.7.2). The extract volume of blood was replaced with sterile saline. The baseline samples were collected at 60 (B1) and 90 min (B2) after the line connection. Thirty minutes after collection of the basal samples, I started with the experimental manipulation (*i.e.*, either the social fear acquisition or infusions of NPS) one by one animal. Subsequently, samples were collected at different time points according to each experimental setup (*see section 2.9*).

2.2.3 Transcardialy perfusion

To fix the brains for immunostaining analysis, I perfused the animals 90 min after behavioral testing, animals were individually transported to another room and deeply anesthetized with CO_2. Following, the thorax was opened, the left ventricle was cannulated, and the right atrium or ventricle was cut to allow efflux of blood. The descending aorta was clamped off. Rats were transcardialy perfused through the brain continuously for about 5 min with 100 ml 0.1 M PBS followed by a 4% paraformaldehyde (PFA) solution (Appendix I) for about 7 min (1ml PFA solution per g of body weight). Brains were then removed and post-fixed in the same fixative at 4°C overnight. Later, brains were immersed in a 30% sucrose solution (Appendix I) at 4°C until the brains sunk to the bottom (±2-3 days). After this, they were snap-frozen in 2-Methylbutan at -80°C. Coronal sections (40 µm) were cut using a cryostat microtome (Leica CM 3050S, Germany). All brain areas were collected in cryoprotection solution (Appendix I) and stored at -20°C.

2.3 **Intracerebral infusions and drug preparation**

To analyze the effects of intracerebroventricular (icv) administration of AVP, NPS, and Cort, rats received a volume of the respective compound (see Table 1) using a Hamilton syringe (Hamilton Bonaduz AG, Switzerland) attached to an infusion system. The applied dose was dependent on each substance and was chosen based on previous studies (see Table 1).

2.4 **Histological verification of the placement of the guide cannulas**

To confirm successful infusion (icv or into the LS) blue ink was infused after euthanizing each animal. For icv experiments, brains were removed and cut with a razor blade at the implantation site of the cannula. If the ventricles were colored blue, the infusion was considered as correct. To confirm the LS infusions, each brain was removed and snap-frozen in 2-Methylbutane (Sigma-Aldrich, Steinheim, Germany) at -80°C. Afterward, brains were cut (40-µm slides) in a microtome (Leica CM 3050S, Germany) and stained with Nissl to localize the cannula tract with the aid of a rat brain atlas (Paxinos and Watson, 1998).

2.5 **Histology of brain tissue**

For c-Fos experiments, coronal sections (40 µm) were cut in a series of four brain slices and storage in a cryo-protected solution (25% Glycerol, 25% Ethylene glycol in 1% PBS) at -20°C. Brain areas were selected following the atlas in (Paxinos and Watson, 1998).

Table 1. Drug information related to the substances used in the pharmacological experiments.
*substances prepared in Ringer solution

Drug	Name	Ip	Icv*	Lateral septum*	Reference	Company
AVP	Arginine vasopressin	-	1ng/5 µl or 0.5ng/5 µl	0.2 ng/0.5 µl	Veenema *et al.*, 2012	Courtesy of M. Manning
V1aR-A	V1a receptor antagonist	-	0.75 µg/5 µl	10 ng/0.5 µl		
NPS	Neuropeptide S (rat) in salt	-	1 nmol/ 5 µl		Slattery *et al.*, 2015	Bachem
NPSR-A	D-Cys(tBu)5-NPS	-	10 nmol/5µl			Courtesy of C. Guerrini
Cort	2-hydroxypropyl-b-cyclodextrin, corticosterone-HBC complex	5 mg/kg (Saline)	10 µg/3 µl		Timmer & Sandi, 2010; Weger *et al.*, 2018	Sigma-Aldrich Chemie GmbH
Met	2-Methyl-1,2-di-3-pyridyl-1-propanone	50 mg/Kg (Saline 60%, propylene glycol 40%)	-		Roozendal *et al.*, 1996; Burman *et al.*, 2010	Sigma-Aldrich Chemie GmbH

2.6 **Behavioral test**

Animal behaviors were manually scored with the program JWatcher version 1.0 (Blumenstein *et al*. 2006) and the distance travelled was automatically tracked using Noldus (Noldus Information Technology, Germany).

2.6.1 Elevated plus maze (EPM)

To measure general anxiety-related behavior I performed the EPM test (see section 1.1.1). The apparatus consists of plus-shaped platform with two open arms (50 x 10 cm, with a rim of 0.5 cm, 40 lux) and two closed arms (50 x 10 x 40 cm, 5-10 lux) connected by a central zone (100 cm^2) and was elevated 80 cm above the ground. If an animal spends less time and perform less entries into the open arms is associated with increased anxiety levels (Pellow et al., 1985). To start the test, the animal was placed facing the closed arm in the central zone. Then, each animal can explore the apparatus for 5 min. A blind observer life scored the animal´s behavior, quantified the entries and time in each zone. According to the percentage of time spent on the open arm (time in open/total arms time*100) the offspring was divided into HAB (less than 10%) and LAB (more than 45%) rats (Liebsch et al., 1998). The apparatus was clean with soapy water between each animal.

2.6.2 Modified Social Preference Test (MSP)

To evaluate the social preference in rats, I modified protocol in (Lukas et al., 2011). Originally the animals were tested during the dark phase, each animal was placed in the center of a grey arena (80 x 40 x 38 cm, 0 lux), after 30 s of habituation, a non-social stimulus (*i.e*. empty wire-mesh cage) was presented in one side of the arena for 4 min. Following, the non-social stimulus was replaced by a social stimulus (*i.e*. an identical wire-mesh cage containing a conspecific of the same sex and weight) and the animal could explore for another 4 min. To provide a pre-training session for the future social fear discrimination phase (*section 2.6.3*) and control the social preference of the animals at the same time, I changed some settings of the protocol. Briefly, in the modified social preference (MSP) version, animals were tested during the light phase (20 lux), each animal was placed in the same arena described above and habituated for 2 min. Afterward, we simultaneously presented the social and non-social stimuli in the extreme sides of the arena. After 4 min of exploration, each animal was returned to its home cage. The arena was cleaned with soapy water (neutral-scented) between each animal. A preference ratio (PR) was calculated as follow:

PR = Social stimulus time – Non-social stimulus time / Total stimuli time.

A positive value that significantly differed from zero indicated social preference.

2.6.3 Social Fear Conditioning (SFC) in rats

As mentioned before, the SFC paradigm was originally developed in mice by Toth et al. (2012) and had to be modified for rats. The following is adapted protocol.

Social fear acquisition phase

One day before social fear acquisition, the animal was placed for one minute into the conditioning chamber for habituation (TSE System GmbH, Bad Homburg, Germany). The conditioning chamber consisted of a transparent Perspex box (45 cm × 45 cm × 22 cm) that was enclosed in a wooden chamber to avoid external noise. Under manually control the TSE software delivers an electric foot shock from the steel grid floor. During social fear acquisition, after 30 s of habituation (Fig.6 A), I presented a non-social stimulus (*i.e.,* an empty wire mesh cage, 20 × 10 × 9 cm) in one corner of the chamber (Fig.6 B). After 3 min, the non-social stimulus was replaced by a social stimulus (*i.e.,* an identical wire-mesh cage containing a conspecific of the same sex and weight). This stimulus will be called as "**Known**" in the next phase (Fig.6 C). Here we split the animals in two groups: **the unconditioned group (SFC^-)** animals that freely explored the social stimulus (3 min) and **conditioned animals (SFC^+)** that received an electric-foot shock (1 mA pulsed current, during approximately 2

seconds) every time they approached the social stimulus. For the latter group, the test lasted at least 7 min after application of one shock, or 5 min, if the animal received more than one shock. Each animal was returned to its home cage thereafter. The chamber was cleaned with mild soapy water (lemon-scented) after each testing.

Retrieval interval is the time between social fear acquisition and social fear discrimination, it varies dependent on the specific memory study in rats, for instance, to test for short-term memory (6 h) or long-term memory (24 h).

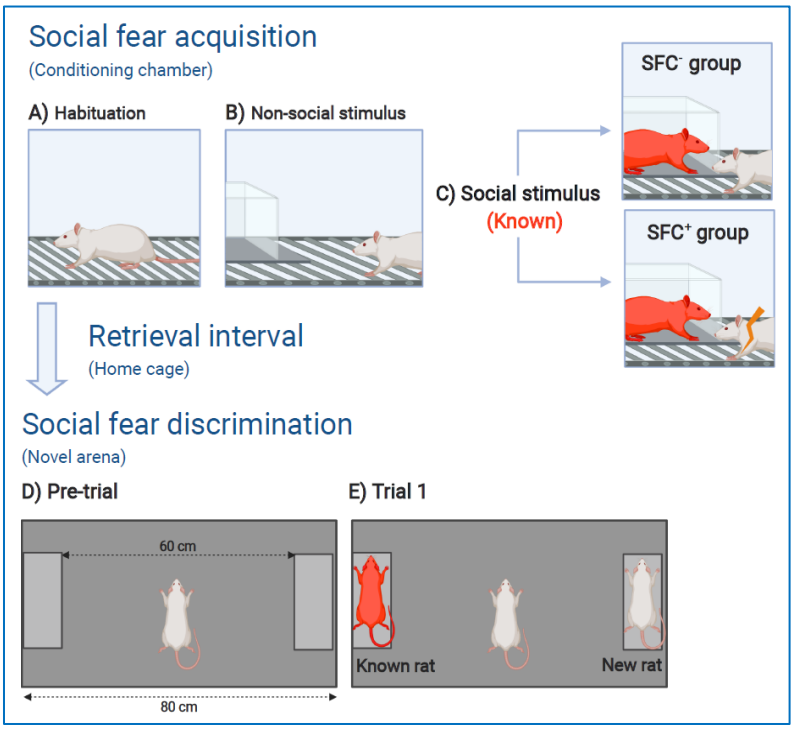


Fig.6. Social fear conditioning protocol in rats. *Social fear acquisition phase:* A) subject is placed in the conditioning chamber to habituate (30s), B) a non-social stimulus presentation during 3 min, C) the latter is replaced by a **social stimulus or Known rat**. Here the animals were split into **conditioned (SFC⁺) or unconditioned (SFC)** groups whether the social investigation is punished or not by an electric foot shock, respectively. Retrieval interval: subject is returned to its home cage. The time in between phases depend on which memory is evaluated: short- (up to 6 h) or long-term (24 h) memory in rats.
Social fear discrimination phase: D) **Pre-trial** subject is habituated to the arena, there is empty cages in the sides to test if the animals fear them. E) **Trial** the Known (used in the social acquisition) and a New stimuli (unknown) are simultaneously present, the subject can explore them for 4 min.

Social fear discrimination phase

To evaluate the level of social fear of the animals, I modified the social discrimination test from (Engelmann et al., 1995). Herewith, I described the changes, first, I placed each animal in the center of an arena (same arena described in 2.6.2) with two empty wire-mesh cages (20 × 10 × 9 cm) on the extreme sides of the arena for a **pre-trial session** (Fig.6. D). This Pre-trial aimed to habituate the

animals to the arena as well to test the non-social fear levels (*i.e*, signs of freezing towards the empty cage). After 4 min of exploration the animal was removed. Then, the empty cages were replaced by one contained the Known stimulus (*i.e.,* used during social fear acquisition phase) and the other contained a **New** stimulus (*i.e.,* an unknown conspecific) (Fig.6 E). In this **Trial session** each animal was returned in the middle of the arena and allowed to free explore both stimuli (4 min). Afterwards, I returned the animal to its home cage. Between animals, the arena was cleaned with soapy water (neutral-scented) and social stimuli were placed into clean wire-mesh cages. I manually scored the investigation time towards each stimulus and calculated the discrimination ratio (DR), as follow:

DR= (New stimulus time – Known stimulus time) / total stimuli exploration time

Here, a positive value significantly different from zero indicated social discrimination (or social recognition). In other words, the animal prefers the New stimulus, while a negative value indicates preference of the Known stimulus.

2.6.4 Cued Fear Conditioning (CFC)

The CFC was performed as previously described by (Slattery et al., 2015) and briefly the protocol consisted of three phases:

Cued fear acquisition (day 1): Animals were placed in the conditioning chamber with the context A (see Table 2), after a 2-min adaptation period, they get exposed to five pairings of the conditioned stimulus (CS; 80 dB, 8 kHz, 30 s) and the unconditioned stimulus (US; 0.7 mA; pulsed current, 2 s). The CS co-terminated with the US, with a 2-min inter-stimulus interval. The animals were returned to their home cage after the last CS-US pairing.

Cued fear extinction (day 2): Rats were placed in context B (Table 2) and, after a 2.-min adaptation period, get exposed to 30 CS presentations without any US, with a 5-s inter-stimulus interval. They were returned to their home cage after the last CS presentation.

Cued fear recall (day 3): Animals were again placed in context B, and after a 5- min adaptation period, they were exposed to 5 CS presentations with a 2- min inter-stimulus interval. Animals were returned to their home cage after the last CS presentation.

Freezing time was automatically measured by the software of the conditioning chamber. For the analysis of extinction data, the CS presentations were pooled into ten points (i.e., the mean freezing percentage of every three CS presentations represented a point) and for the recall data, I analyzed the mean freezing percentage of the five CS presentations.

Table 2. Characteristic of each context in the CFC

	Context A	Context B
Size	45× 45× 40 cm	45×45×40 cm
Color	Transparent	black
Cleaning detergent	lemon-scented	neutral-scented
Texture	electric grid floor	smooth floor
Illumination	50 lux	10 lux

2.6.5 Hargreaves' Plantar Test

The Hargreaves' Plantar Test was used to test for differences in pain perception between HAB, LAB and NAB lines, in a similar manner described by (Jochum et al., 2007). Animals were habituated to the Plexiglas box on the glass floor of the test equipment (Ugo Basile model 7371, Italy) 5 min before the test started. Subsequently, a focused thermal heat stimulus was delivered from a fixed distance to the plantar surface of the hind paw, and paw withdrawal latency was measured. Each of the hind paws was tested thrice with a 2 min interval between measures in the same paw. Data represent an average of 6 trials per animal.

2.7 **Molecular techniques**

2.7.1 Analysis of mRNA expression

RNA isolation and reverse transcription

Total RNA of the LC, PVN, and AMY was isolated using peqGOLDTriFast (Peqlab, Germany) according to the manufacturer´s protocol. To prevent RNAse activity, the isolation was carried out on ice. Additionally, all instruments were cleaned with RNase degradation solution (RNase Zap, Ambion, USA). The tissue probes were homogenized in 1 ml Tri-reagent and incubated at room temperature (RT) for 5 min. Next, 200 µl Chloroform were added, vortexed and centrifuged (17000 x g, 4 °C, 20 min). The upper aqueous phase containing RNA was carefully transferred into a new cup. Subsequently, 500 µl Isopropanol (equal to 45%) and 1 µl Glycogen (RNA grade; Thermo Scientific, Germany) was added and incubated overnight at -20 °C to precipitate the total RNA. On the next day, samples were centrifuged (17000 x g, 4 °C, 30 min) and the supernatant was discarded. The RNA pellet was washed twice with ice-cold 80 % ethanol. After air drying for 5-10 min, the RNA pellet was eluted in 15 µl RNAse free H2O (DEPC, Carl Roth) and incubated at 70 °C, 1.000 rpm for 5 min in a heating block (Thermomixer compact, Eppendorf, Germany). The RNA concentration and

absorbance ratios at 260/280 nm and 260/230 nm were assessed using a NanoDrop spectrophotometer (ND-1000; Thermo Scientific).

For reverse transcription of RNA into complementary DNA (cDNA), 1µg of isolated total RNA was incubated with 1 µl of random primers (Life Technologies), 1 µl of a dNTP mix (10 mM each base; Invitrogen, Germany), and 1µl RNAse-free H_2O. To anneal the primers, the mixture was incubated for 5 min at 65 °C (Mastercycler nexus X2, Eppendorf, Germany). In the next step, 4 µl of 5x First Strand Buffer (Thermo Scientific), 1 µl of 0.1 M dithiothreitol (DTT; Invitrogen, Thermo Scientific) and 1 µl of RNase OUT™ (40 U/µl; Life Technologies) were added to the sample and mixed (+RT samples). To test contamination of genomic DNA, we transferred 2 µl of the total volume in new cups. These –RT samples served as negative controls in the following qPCR. Subsequently, for the synthesis of cDNA, 1 µl reverse transcriptase Super ScriptTM IV (200 U/µl; Invitrogen, Thermo Scientific) was added to the +RT samples. The synthesis of cDNA was performed at 50 °C for 10 min followed by the inactivation of the enzyme at 80 °C for 10 min.

Quantitative real-time PCR (qPCR)

Primers were designed using Primer-BLAST (open-source software NIH) (Table 3). Specificity of the selected primer pairs was tested using regular qPCR.

Table 3. Evaluated primers

Candidate gene	Primer sequence (5´-3´)
GAPDH	fwd TGATGACATCAAGAAGGTGG
	rev CATTGTCATACCAGGAAATGAG
NPS	fwd CCGGTCCTCTCTTCCAAGGT
	rev TGGATTTGGGCACTCCACC
NPSR	fwd GTGGGGCTCCTTCTACTCGT
	rev CTCTTTCAGCTCTCTCCAGTCC

To validate the selected primers and quantitatively analyse mRNA expression changes qRT-PCR was conducted for various genes (Table 3) using the QuantStudio™ 5 Real-Time PCR System (Thermo Fischer, Germany). The DNA intercalating fluorescent SYBR Green QuantiFast (PowerUp™ SYBR™ Green Master Mix, Applied Biosystems) served as detection dye. 2 µl of cDNA was added (or H2O as negative control, or –RT sample to check for genomic contamination) to the reaction mixture, which consisted of 9µl nuclease-free H2O, 2 µM forward and reverse primer, and 5 µl SYBR Green.

All samples were pipetted in duplicates into a 96 well plate, which was covered with foil and centrifuged at 1.000 x g at RT for 1 min. The thermal cycler monitored the fluorescence signal as amplification occurred during the reaction progress (Table 4). Subsequently, samples were slowly heated from 50 °C to 95 °C to analyze primer specificity using the created melt curve. mRNA expression of the selected genes was calculated in relation to the housekeeper glyceraldehyde-3-phosphate dehydrogenase (GAPDH)using the delta-delta CT method.

Table 4. Steps of qPCR.

qPCR step	Temperature (°C)	Duration (s)	Cycles
UDG activation	50	120	Hold
Initiation/DNA	95	120	Hold
Denaturation	95	3	40x
Annealing/extension	60	30	

*Denaturation and annealing/ extension steps were repeated for 40 cycles

2.7.2 Analysis of plasma samples (Cort, NPS and OXT)

Blood samples were centrifuged at 5000 g for 15 min at 4 °C. Blood plasma (supernatant) was transferred into new cups and stored at -20 °C. Plasma Cort was measured using the corticosterone Enzyme-linked immunosorbent assay (ELISA; IBL international GmbH) according to manufacturer´s instructions. The plasma samples were diluted 1:10 using phosphate buffered saline (1 x PBS; no difference to the original Standard 0). Subsequently, 20 µl of each standard, control, and samples were pipetted into the respective wells. All samples were analyzed in triplets. Following, 200µl of enzyme conjugate was added to each well and the plate was incubated at RT for 1h with gentle agitation. After that, 3 washing steps were performed with the washing buffer and 100µl of the substrate solution was added to each well. After 15 min incubation at RT with constant shaking the enzymatic reaction was stopped with 50 µl of stop solution. An OPTIMA plate reader (BMG Labtech GmbH) was used for the analysis of Cort concentrations at a wavelength of 450 nm within 10 min after adding the stop solution. Concentrations of Cort were evaluated with the help of the MARS Data Analysis Software (BMG Labtech GmbH). From a sample of 100 µl, the NPS levels were measured by the Elisa kit (DEIA-AH063, Creative Diagnosics) following manufacturer´s instructions. OXT levels were measured via radioimmunoassay (RIAgnosis, Germany).

2.7.3 cFos immunohistochemistry

40 µm thick coronal brain sections (4 brain slices per animal, per well in a 12 well plate) were washed 3 times 15 min in 1x PBS with a pH of 7.4 at RT. To block the endogenous peroxidase activity, sections were treated with 3% H_2O^2 in 1x PBS for 15 min (Appendix I). After another three 15-min washing steps in 1x PBS, unspecific binding was blocked using PBST/1% NGS solution (Appendix I) for 1 h at RT. Subsequently, they were incubated with anti-c-Fos antibody (1:2000, diluted in blocking solution, table 5) for 1 h at RT followed 48 hours at 4°C and continuous shaking. Subsequently, the sections were rinsed 3 times for 10 min in 1x PBS and incubated with a biotinylated goat anti-rabbit IgG secondary antibody (1:1000, diluted in PBST/1% of NGS solution, Table 5) for 2 h at RT. After another 3 washing steps (1x PBS), an avidin-biotin solution was added for 1 h at RT (prepared 30 min before use, following Vector Laboratories protocol; see Appendix I) and slices were washed again 3 times for 10 min in 1x PBS. To visualize c-Fos positive cells, 3,3'-diaminobenzidine (DAB) staining was performed using the DAB Substrate Peroxidase (HRP) intensified with Nickel (SK-41000, Vector Laboratories, USA; see Appendix I). DAB is reacting as a chromogen in the presence of the HRP enzyme and produces a brown reaction. The reaction was stopped after 1 min since an observable background staining occurred. Finally, the slices were washed 6 times for 10 min in 1x PBS and mounted on superfrost microscope slices. The slides were dried overnight and covered using Roti-Histokitt (Appendix I)

Table 5. List of antibodies

Name	Company	Product number
anti-c-Fos antibody, Rabbit polyclonal to c-Fos	abcam, Cambridge, UK	ab 190289
goat anti-rabbit IgG secondary antibody, biotinylated	Vector Laboratories, Burlingame, CA 94010 USA	BA-1000

2.8 **Image analyses for C-fos quantification**

Brain slice pictures of HP and AMY were taken with a microscope (Leica, DFC9000 GT) and processed with Fiji (www.imagej.net/Fiji). I divided the dorsal HP into subregions: CA1, CA2/3 and DG (Fig. 7.A and B) to count for each hemisphere. As well, AMY was divided into subregions: BLA, CeA and MeA (Fig. 7.A and D). C-Fos-positive cells were identified as round and dark cells (Fig. 7.C and E). Statistical analysis was done based on the average of four slices per animal.

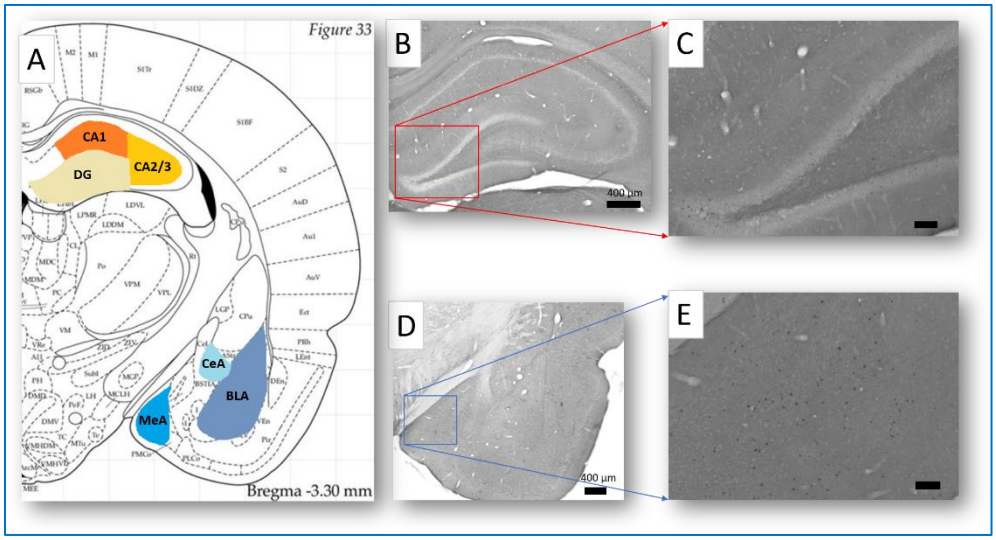


Fig. 7. c-Fos-positive cells staining pictures. A) Anatomical subregions used as guide for the dorsal hippocampus (dHP) and amygdala (modified image form Brain Atlas XX (Paxinos and Watson, 1998)). B) dHP view (25X) and its magnification detail (C; 100X). D) Amygdala overview (25X) and its magnification detail (E, 100X).

2.9 **Experimental protocols**

Part I Social fear conditioning in rats

2.9.1 Establishment of social fear conditioning in rats

Protocol settings in the SFC adapted to rats

The establishment of the SFC paradigm for rats was performed in NAB rats and required substantial modifications. For instance, I tested different shock intensities, as well the best context to measure the social fear (*i.e.,* home cage *vs* novel arena and light *vs* dark period). Among other features that are describe in detail in this section.

Retrieval interval effects

Using the adapted protocol to rats, I evaluated how different retrieval intervals (at 2, 4 and 6 h) affects the social fear response in rats.

Specificity of the induced social fear

To assess whether the specificity of the CS-US pairings affected the social fear response, I included different control groups (I always used an electric foot shock as US, the CS vary in each control). First, all animals were habituated to the conditioning chamber for 30 s, after this, they were split into the following groups:

Shock group (N=9) received four electric foot shocks (1 s, 1 mA, *i.e*. the average number received during social fear conditioning) with a 30 s inter-shock interval. After the last shock, the animals were kept in the conditioning chamber for 1 min before they were returned to their respective home cage. This group evaluates whether repeated exposure to electric shocks alone is enough to alter the social fear response in rats.

Unpaired group (N=10) could explore the empty cage for 3 min. Then, the empty cage was replaced with a social stimulus, and 4 shocks were delivered randomly independent of the occurrence of social exploration. Thereby, I aimed to determine whether the presence of a social context without the pairing of an US leads to social fear.

Non-social group (10) received shocks delivered at 10, 30, 40 s, and 1 min in presence of the empty cage (used in the original protocol as non-social stimuli). It aimed to control if the empty cage is perceived as a cue for contextual fear.

Unconditioned (SFC^-) group (N=9) could freely explore an empty cage and a social stimulus without any shocks to control for normal social investigation.

After 6h, all groups were tested in the social fear discrimination phase as described in the section 2.6.3.

2.9.2 Responses of HAB, LAB and NAB rats in the SFC paradigm

Behavioral characterization

First, all animals were tested in the EPM (see 2.6.1) to confirm their trait anxiety level. Three weeks later, they were tested in the social preference test (Lukas et al., 2011) as a control for normal social behavior. After another week they were divided into SFC^+ and SFC^- groups during SFC. Social fear discrimination was performed after 24 hours (see 2.6.3). Additionally, all animals were tested in the CFC (see 2.6.4) and in Hargreaves' Plantar Test (HT, see 2.6.5) to determine any impairment in fear learning or differential pain sensitivity, respectively (each test were done with one week of separation in between).

2.9.3 Role of AVP in long-term social fear memory

AVP is well known to play a key role in the regulation of social memory (see 1.3.3). As previously mentioned, HAB rats have a higher activation of the AVP system due to a single nucleotide polymorphism in the promoter of the AVP gene. Thus, I hypothesized that AVP mediates the long-term and individual social memory in HAB rats, and additionally may promote social recognition in LAB and NAB rats.

Central effects of AVP in the social fear consolidation

To investigate, whether brain AVP modulates social fear consolidation in HAB, LAB and NAB rats. I implanted a cannula above the lateral ventricles to deliver substances (see 2.2.1). After three days of recovery, rats underwent social fear acquisition and were immediately thereafter I centrally infused either AVP, AVP antagonist (V1a-A) or Ringer solution (vehicle group: Veh). Applied doses are described in the section 2.4. After 24 hours, the social fear discrimination was assessed.

Effects of AVP in the lateral septum during social fear consolidation

Therefore, to investigate the potential role of AVP within the LS in social fear consolidation and individual discrimination in HAB and LAB rats, animals were implanted with a guide cannula above the LS (see details in 2.2.1). After four days of recovery, animals underwent social fear acquisition and were immediately thereafter bilaterally infused with either AVP, V1a-A, or Ringer solution into the LS (see section 2.3). After 24 hours, the social fear discrimination was assessed.

2.9.4 Effects of GC in social fear consolidation

Next, I tested the hypothesis whether GC modulate long-term consolidation of social fear memory. As HAB rats also showed a hyperactive HPA axis response and high Cort levels which, in turn, may act as an enhancer of memory consolidation. First, I measured plasma Cort levels by ELISA along with the different phases of SFC to compare between lines (HAB vs NAB) and within lines (SFC^+ vs SFC^-). Second, I blocked the Cort availability in HAB and LAB rats before the social fear acquisition to see how their memory consolidation change. Finally, I administrated Cort to NAB rats to prove if the social fear memory can be enhanced from 6 to 24 h, either with peripheral or central Cort administration. Doses are described in *section 2.3*.

2.9.5 Brain activation during the social fear discrimination

To reveal differences in brain region-specific neuronal activation between HAB and NAB rats, which might contribute to the long-term social fear memory observed in HAB rats, I quantified the c-Fos positive cells after social fear discrimination (*sections 2.5; 2.7.3, 2.*8). Briefly, HAB and NAB rats were tested first in the EPM followed by the MSP. Animals were split into SFC^- and SFC^+ groups within each line; and SFC^+/NAB rats were split into two additional groups following acquisition to compare the short- (after 6 h) *vs* long-term memory (after 24 h). Ninety min after the end of the test, the animals were sacrificed and perfused (*see 2.3.3*) to investigate if the AMY or HP were differentially activated between (HAB *vs* NAB) and within breeding lines (SFC^+ *vs* SFC^-).

Part II NPS contribution to the behavioral and stress response in virgin and lactating females

2.9.6 Basal mRNA expression of NPS and its receptor in PVN, AMY and LC

To compare basal expression levels of NPS and its receptor in selected brain areas between virgin and lactating (PND 3-5) females, rats were sacrificed by decapitation, brains were rapidly removed, snap-frozen in 2-Methylbutane (Sigma-Aldrich, Steinheim, Germany) at -80° C and stored at -80°C. The brains were cryo-sliced (300 µm) in a microtome (Leica CM 3050S, Germany) to obtained 3-4 consecutive slides from PVN, AMY and LC (following (Paxinos and Watson, 1998)). Each slide was placed on chilled plates and under a stereomicroscope; the tissue was dissected using a punching needle (1 mm diameter). The obtained pellets were processed to quantify the mRNA by qPCR, see details in *section 2.7.1.*

2.9.7 Central effects of NPS on cued fear extinction

I aim to evaluate the effects of NPS or an NPSR-A on cued fear extinction and plasma Cort responses in virgin and lactation female rats. Briefly, animals were implanted with a cannula above the lateral ventricles (*see 2.2.1*). After three days of recovery, rats were conditioned for cued fear (*see 2.6.4*) and received an infusion (icv) of either NPS, NPSR-A or Veh (*see 2.3*) 20 min before the extinction phase. Two days after the recall phase they received a second icv infusion (each animal received the same treatment as in their first infusion) and after 20 min were decapitated to collect trunk blood (see 2.2.2). Vaginal smears were taken every day after the experiment to classify the estrous phase of each virgin female.

2.9.8 Effects of NPS on Cort release under basal and stress-induced conditions

To measure plasma Cort responses to icv NPS or its antagonist (*see 2.3*) female rats were implanted with a jugular vein catheter (see 2.2.2) as well as a guide cannula above the lateral ventricles (see *2.2.1*). Lactating dams received the double surgery on the PND-1, and for each experimental cohort, I included virgin rats (same age and isolation time than dams) as controls. After four days of recovery, I connected the jugular vein catheter to a line with heparinized saline following a similar protocol described above (*see 2.2.2*). Baseline samples (0.3 ml each sample) were collected at 60 (B1) and 90 min (B2) after connection of the lines. Afterwards, I infused an animal every 5 min and collected samples after 15, 25, 35 and 90 min after the infusion time. Moreover, right after taking the sample at 15 min after infusion, each animal was placed in an elevated platform (20 cm diameter

and 70 cm elevated from the floor) for 5 min and returned to the home cage to take the next blood sample. Blood samples were processed as described before (*see 2.7.2*).

2.10 **Statistical Analysis**

Statistical tests were performed using SPSS, version 20 (IBM, Germany). Graphics were made with GraphPad Prism, version 8.0 for windows (GraphPad software, USA). Only rats with correctly implanted cannulas were included in the statistical analysis. Statistical assumptions of normality and homoscedasticity for Analysis of variance (ANOVA) and Student´s T-test were checked with Shapiro-Wilk test and Brown-Forsythe test, respectively. Otherwise, I used a Welch´s T-test or non-parametric analysis accordingly. Data was presented as the mean ± standard error (SEM). $p < 0.05$ was considered statistically significant.

Results

3. Results

Part I: Social Fear Conditioning in rats

The first aim of my thesis was to establish the SFC protocol in rats. In this regard, the original protocol for mice had to be adapted to rats which required different settings. Most of these changes were based on intrinsic differences between these two species. The modifications are listed in *section 3.1.* After the successful establishment of the SFC protocol, I combined the SFC with the HAB-LAB model to determine the vulnerability to acquire long-term social fear memory (*i.e*., 24 h after social fear acquisition) in the three rat lines (HAB, LAB, and NAB), results are shown in *section 3.2.* The following experiments evaluated the effects of AVP (*section 3.3*) and GC (*section 3.4*) in the social fear consolidation. Finally, I performed a cFos analysis comparing the neuronal activation of the HP and the AMY during the social fear discrimination within SFC^+ and SFC^- in NAB and HAB rats, after 6 h and 24 h, respectively.

3.1 **Establishment of Social Fear Conditioning in rats**

3.1.1 Protocol settings of the SFC adapted to rats

The SFC protocol required some modifications to properly evaluate social fear and its extinction in rats, which I outline below. In addition, I included a series of pilot studies that I conducted to determine an optimal setup of the SFC protocol for rats.

<u>Social fear acquisition settings</u>

Pre-habituation to conditioning chamber: 24 h before the social acquisition phase, I included a short session of habituation (1 min) with the rats in the conditioning chamber. This also facilitated the exploration of the stimuli (*i.e*., non-social, and social stimuli) during the social fear acquisition phase.

Shock intensity: during the establishment of the protocol, I tested different shock intensities (from 0.7, 0.8, to 1 mA) in the social fear acquisition. Although, no formal study has compared them directly, here, I illustrate the effects of two shock intensities, *i.e.* 0.7 and 0.8 mA. The data belong to two experimental pilots that were used to additionally evaluate other settings, such as the effects of light-dark cycle in the SFC and the context of the social fear extinction (*i.e*., evaluate the extinction in the homecage *vs* novel cage) addressed in the next sections. Using 0.7 mA, I did not find any

difference in the CS-US pairings between animals conditioned during light *vs* dark period (Fig.8 A, Table 6). Moreover, using 0.8 mA, no differences in CS-US pairings were found for animals which were tested for the context during social fear extinction (Fig.8 C, Table 6). Similar, when I compared the CS-US pairings between these two shock intensities (0.7 *vs* 0.8 mA) no significant differences were observed (H=3.51; p=0.319). More importantly, neither 0.7 nor 0.8 mA induced significant differences in the investigation time between SFC^+ *vs* SFC^- (Table. 6, Fig.8. B and D), revealing that both intensities are too low to lead to a proper acquisition of social fear. Therefore, for the final protocol, I selected an intensity of 1 mA, which promoted a robust social fear response in the SFC^+ groups (data shown in *section 1.1.2* and remaining parts of the thesis).

Table. 6. Statistical analysis of the experimental pilots to assess effects of Light-Dark cycle and context on social fear extinction (Fig. 8).

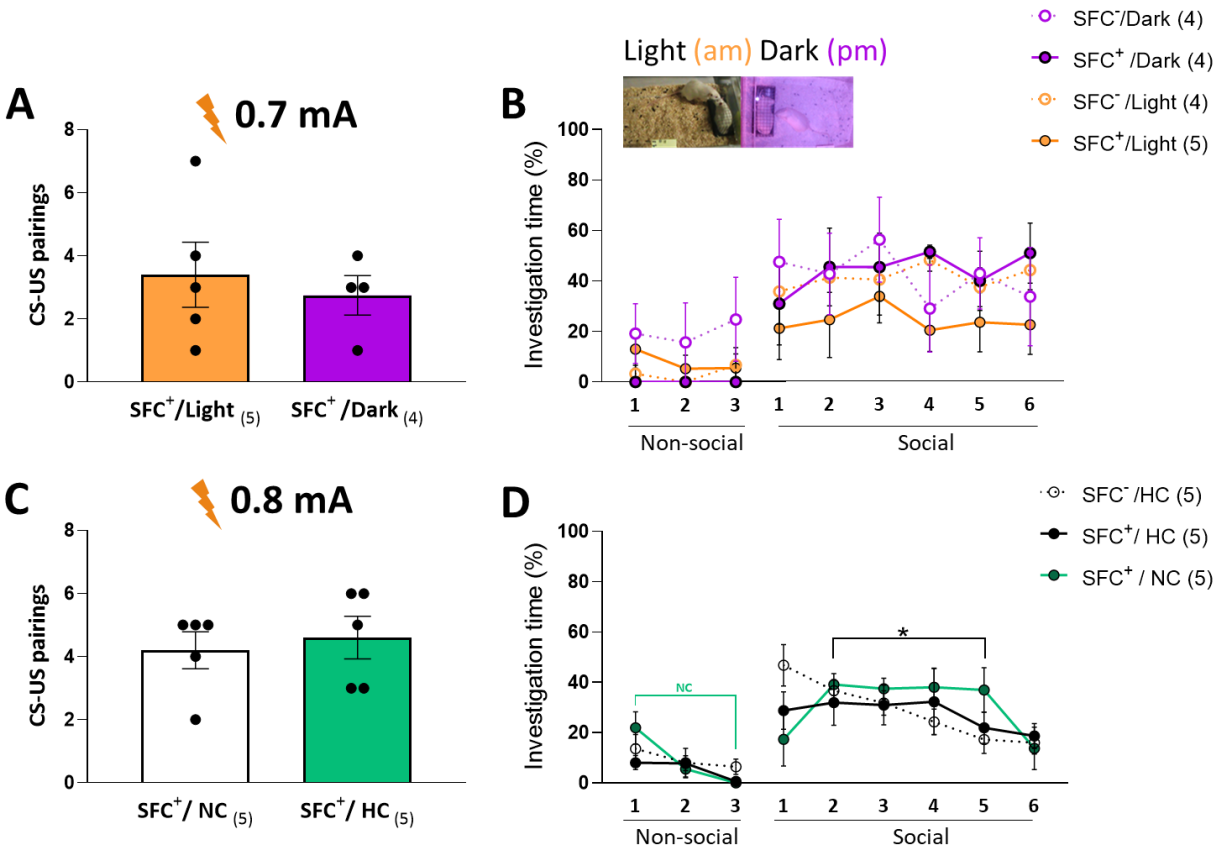
Experiment: Dark-light cycle effects / Shock intensity 0.7 mA				
Test	**Dependent variable**	**Factor (levels)**	**Statistical**	***p* value**
SFC Acquisition	CS-US pairings (Student´s T-test)	SFC^+ (SFC^+/Dark *vs* SFC^+/Light)	t_7=0.50	0.630
SFC Extinction	Non-social investigation (%) (Two-way ANOVA for repeated measures)	Groups	$F_{(3, 13)}$ = 1.19	0.350
		Time (ns1-ns3)	$F_{(1,864, 24,23)}$ = 1.97	0.163
		Groups x Time	$F_{(6, 26)}$ = 1.12	0.377
	Social investigation (%) (Two-way ANOVA for repeated measures)	Groups	$F_{(3, 13)}$ = 0.91	0.461
		Time (s1-s6)	$F_{(2.683, 34.88)}$ = 0.58	0.613
		Groups x Time	$F_{(15, 65)}$ = 0.60	0.862
Experiment: Context of extinction (Homecage *vs* New cage) / Shock intensity 0.8 mA				
Test	**Variable**	**Factor (levels)**	**Statistical**	***p* value**
SFC Acquisition	CS-US pairings (Student´s T-test)	SFC^+ (SFC^+/HC *vs* SFC^+/NC)	t_8=0.44	0.666
SFC Extinction	Non-social investigation (%) (Two-way ANOVA for repeated measures)	Groups	$F_{(2, 12)}$ = 0.51	0.612
		Time (ns1-ns3)	$F_{(1.699, 20.39)}$ = 11.15	0.001***
		Groups x Time	$F_{(4, 24)}$ = 2.41	0.076
	Social investigation (%) (Two-way ANOVA for repeated measures)	Groups	$F_{(2, 12)}$ = 0.07	0.9264
		Time (s1-s6)	$F_{(2.812, 33.75)}$ = 5.24	0.005**
		Groups x Time	$F_{(10, 60)}$ = 2.67	0.009**

Social fear discrimination settings

Light period to measure social fear in rats

Rodents are nocturnal, and this may play an important role to study social behaviors in the laboratory. To select the best time point for the social fear evaluation in rats, I assessed whether the light (at 08:00) or dark (at 20:00) period had an impact on social fear extinction. During social

fear extinction, no significant differences were observed between groups neither in the non-social nor social investigation percentage (Table 6, Fig. 8 B). Based on these data, I choose to perform all



further experiments during the light period.

Fig.8. Pilot experiments used to establish the social fear conditioning (SFC) protocol in rats non-selected for anxiety-related behavior. Upper part of the figure: Experiment to test the effects of the light-dark cycle on extinction of social fear during the SFC paradigm. A) CS-US pairings (number of shocks; shock intensity: 0.7 mA) of conditioned rats (SFC^+) during acquisition of social fear, which were later tested during the light or dark period. Unconditioned rats (SFC^-) received no shocks. B) Investigation time (%) during social fear extinction (*i.e.*, serial 3-min presentation of three non-social stimuli, followed by six social stimuli, with an interval of 3 min between presentations). Bottom part of the figure: Experiment to evaluate the effects of context effects in the social fear extinction, *i.e.*, animals tested in the homecage (HC) versus animals tested in a new cage (NC). C) Number of shocks (CS-US pairings; 0.8 mA intensity) during acquisition of social fear. D) Investigation time (%) during social fear extinction. ^{NC}p=0.056 indicated that in SFC^+/NC group the non-social stimuli exploration decreases over time., * p<0.05 indicated in the SFC^-/HC group that social exploration decreases over time. Data represent mean ± SEM, group sizes are given in parentheses.

Context to measure social fear in rats

Exposure to a new environment may increase the level of exploration of the animals compared to remaining in the home cage (HC). However, this could also have the opposite effect, as novelty can also induce anxiety-like behaviors and delay the exploration (Litvin *et al.*, 2008). Therefore, I

performed a pilot study to evaluate the effects of context. Animals were split in three groups: unconditioned animals (SFC^-/HC) as control, conditioned animals that remained in the home cage (SFC^+/HC) for the social fear extinction and conditioned animals that were measured in a new cage (SFC^+/NC).

SFC^+ groups (HC versus NC) did not differ in the CS-US pairings (Table 6, Fig.8. C), reflecting similar social fear acquisition. Moreover, during the social fear extinction, no differences in the time of non-social or social investigation were observed between all groups (Table 6, Fig.8.D). However, there was a significant main effect of time in non-social and social presentations (Table 6). *Post hoc* analysis indicated that the SFC^+/HC group showed a trend towards decreased exploration of the non-social stimuli over time (ns1 *vs* ns3; p=0.051). In addition, only SFC^-/HC rats showed a decrease in the social exploration over time (s2 *vs* s5; p=0.037). Lastly, in response to other relevant modifications of the protocol, such as stimuli used and stimuli presentation (for details see the next sections), the context of the test was again modified. In this regard, I selected a bigger arena (40 x 80 cm) that enabled the measurement of social fear discrimination between the social stimuli (Known *vs* New stimulus, with a distance between stimuli of 60 cm). A detailed description of the animal response to this specific context can be found in the *section 1.1.2* and the remaining experiments in part I of the thesis.

Stimuli used in the test

Presentation of the **Known stimulus**, *i.e.*, the conspecific used during social fear acquisition, to examine social fear expression in rats seems to promote a more robust social fear response. Thus, for the final protocol I always present the Known stimulus, a major difference with the mouse protocol, where a series of new conspecifics are presented during extinction of social fear.

Stimuli presentation

The observed higher fear expressed in the presence of the Known stimulus implicated a social recognition component as crucial factor for proper social fear. Therefore, I replaced the “serial” presentation of the social stimulus (*i.e.*, one stimulus at a time for 3 min, 3 min-inter-stimulus interval) by a simultaneous presentation of two social stimuli. I based this setup on a modified version of the social discrimination paradigm of Engelmann et al. (Engelmann et al., 1995). Here, the subject can explore the Known and a New stimulus (both of same sex and age as the experimental subject) for 4 min. Considering the innate drive to investigate an unfamiliar conspecific more than a

familiar one (Camats Perna and Engelmann, 2017), a preference for the New stimulus accompanied by fear behaviors, such as freezing, is operationalize as social fear memory. Moreover, there should be a reduction of the overall social investigation in the SFC^+ compared to the SFC^- group, as seen in mice, to consider each subject successfully conditioned.

Absence of the empty cage during the consolidation period

Originally, in the mice protocol, animals were exposed to an empty cage overnight. However, I decided not to expose the rats to the empty cage overnight, the reason behind this decision is discussed in *section 4.1*.

3.1.2 Retrieval interval effect on social fear memory

So far, the different SFC pilots experiments in NAB rats failed to promote long-term social fear memory (24 h). In contrast, to the consistent long-term fear responses in mice using similar settings (for review see Masis-Calvo *et al.,* 2018). This resembles the interspecies differences already described in social memory (*i.e.,* neutral memory usually measured with juvenile as stimuli). The ability of mice to maintain social memory up to 24 hours has been widely replicated, while in rats social memory has only been reported to last up to 45 min in males and 2 h in females (Camats Perna and Engelmann, 2017). Nonetheless, in my experiments I observed that few rats showed social fear after 24 (Fig.8.B), suggest that they can learn a prolong the memory although they do not retain it well. Therefore, I hypothesized that rats indeed acquire social fear memories, but their expression is limited to shorter retrieval intervals. Hence, I evaluated the effect of three retrieval intervals on social fear memory in NAB rats, specifically 2, 4, and 6 h after social fear acquisition.

During social fear acquisition, SFC^+ groups (2 h *vs* 4 h *vs* 6 h) did not differ in the number of CS-US pairings (Table 7), reflecting similar social fear learning. On average, all SFC^+ rats needed 2.8 ±1 electric foot shock before they stopped to approach for at least five min. During social fear discrimination, I observed a significant effect of SFC (*i.e*., SFC^+ *vs* SFC^-), but neither a main effect of the retrieval interval (*i.e.*, 2 h *vs* 4 h *vs* 6 h) nor interaction between factors (SFC x Retrieval interval) in all the variables measured (Table 7). *Post hoc* tests revealed that all SFC^+ animals showed significantly reduced social investigation (Fig.9. A-C) and increased freezing time (Fig.9. D-F) compared with the SFC^- groups in each retrieval interval (see *p* values in Table 8). In addition to fear expression, I assessed the discrimination ratio (Table 8). Here, a significant positive value indicates intact social memory since animals naturally prefer to explore a New over a Known conspecific.

Curiously, SFC$^-$ animals discriminated at 2 and 6 h (Table 8, Fig.9. G and I), but not at 4 h (Table 8, Fig. 9.H). In contrast, none of the SFC$^+$ animals displayed social discrimination.

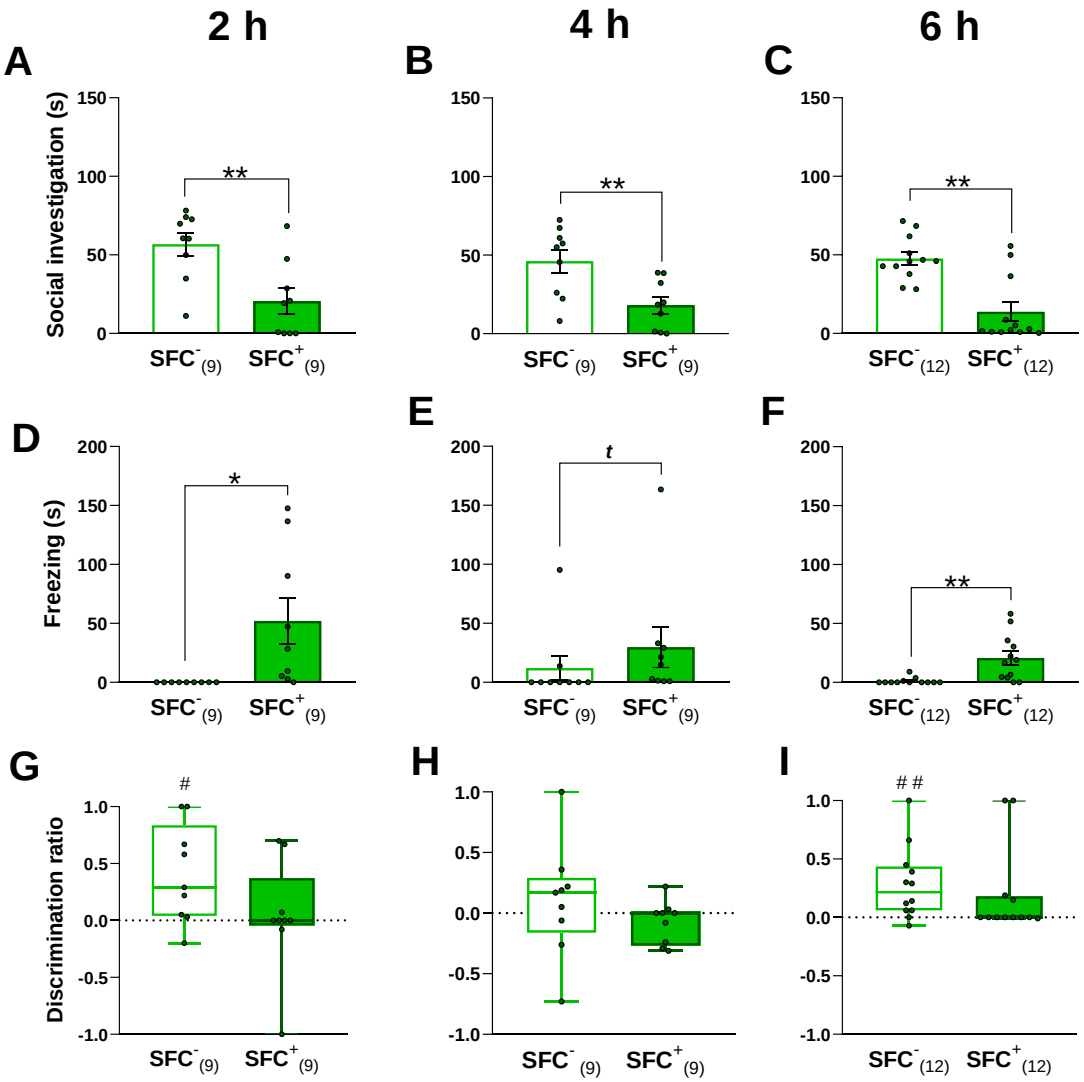


Fig. 9. Behavioral response during social fear discrimination at different retrieval intervals (2h, 4h, 6h) in male rats non-selected for anxiety-related behavior (NAB). A-C) Social investigation time ($^{**}p$<0.01, SFC$^-$ vs. SFC$^+$ in each interval) and D-F) Freezing time (SFC$^-$ vs. SFC$^+$: $^{*}p$<0.05, ^{t}p<0.07). G-I) Discrimination ratios ($^{\#}p$<0.05 or $^{\#\#}p$<0.01 indicated significant difference from critical value zero (*i.e.*, preference is not by chance), positive value indicated social memory). Data represent mean ±SEM, sample size for each group is noted in parentheses.

Table. 7. Statistical analysis of effects of retrieval interval in the social fear extinction.

Test	Variable	Factor (levels)	Statistical	*p* value
SFC Acquisition	CS-US pairs (Kruskal-Wallis-test)	SFC^+	H=4.28	0.117
SFC Social fear discrimination (Fig. 9)	Social investigation (s) (Two-way ANOVA)	SFC (SFC^+ *vs* SFC^-)	$F_{(1, 44)}$ = 31.52	0.001***
		Retrieval Interval (2 h *vs* 4 h vs 6 h)	$F_{(2, 44)}$ = 0.71	0.496
		SFC x Retrieval Interval	$F_{(2, 44)}$ = 0.19	0.829
	Freezing (s) (Two-way ANOVA)	SFC (SFC^+ *vs* SFC^-)	$F_{(1, 44)}$ = 7.92	0.007**
		Retrieval Interval (2 h vs 4 h vs 6 h)	$F_{(2, 44)}$ = 0.85	0.434
		SFC x Retrieval Interval	$F_{(2, 44)}$ = 1.26	0.294

Table 8. Comparisons between SFC^- and SFC^+ groups during the social fear discrimination with different retrieval interval (Fig. 9 and 10).

Behaviors	2 h	4 h	6 h
Social investigation (s) (Student t-test)	t_{16}=3.33 p=0.004**	t_{16}=3.07 p=0.007**	t_{22}=4.70 p=0.0001***
Freezing (s) (t-test with Welch´s correction)	*Welch's* t_8=2.66; p=0.028*	*Welch's* $t_{8.79}$=2.24; p=0.053^t	*Welch's* $t_{11.41}$=3.34; p=0.006**
Discrimination ratio in SFC^- (Wilcoxon-test)	p=0.019$^{\#}$	p=0.496	p=0.005$^{\#\#}$
Discrimination ratio in SFC^+ (Wilcoxon-test)	p>0.999	p=0.218	p=0.125
Pre-trial distance travelled (Student t-test)	t_{16}=0.48 p=0.634	t_{16}=1.31 p=0.207	t_{12}=0.19 p=0.846
Trial distance travelled (Student t-test)	t_{16}=2.24 p=0.039*	t_{16}=1.92 p=0.073^t	t_{12}=3.101 p=0.009**

Moreover, computerized tracking of the animal's locomotion showed that during the pre-trial period (*i.e.*, exploration of the arena with two empty cages), no significant differences in the distance traveled were found between the different SFC^+ and SFC^- groups (Table 8, Fig.10. A-C). In contrast, during the trial period (*i.e.*, during social stimuli presentation), all SFC^+ groups (2 h, 4 h, 6 h retrieval interval) showed a significant reduction of the distance traveled compared to the SFC^- groups (Table 8, Fig.10. D-E).

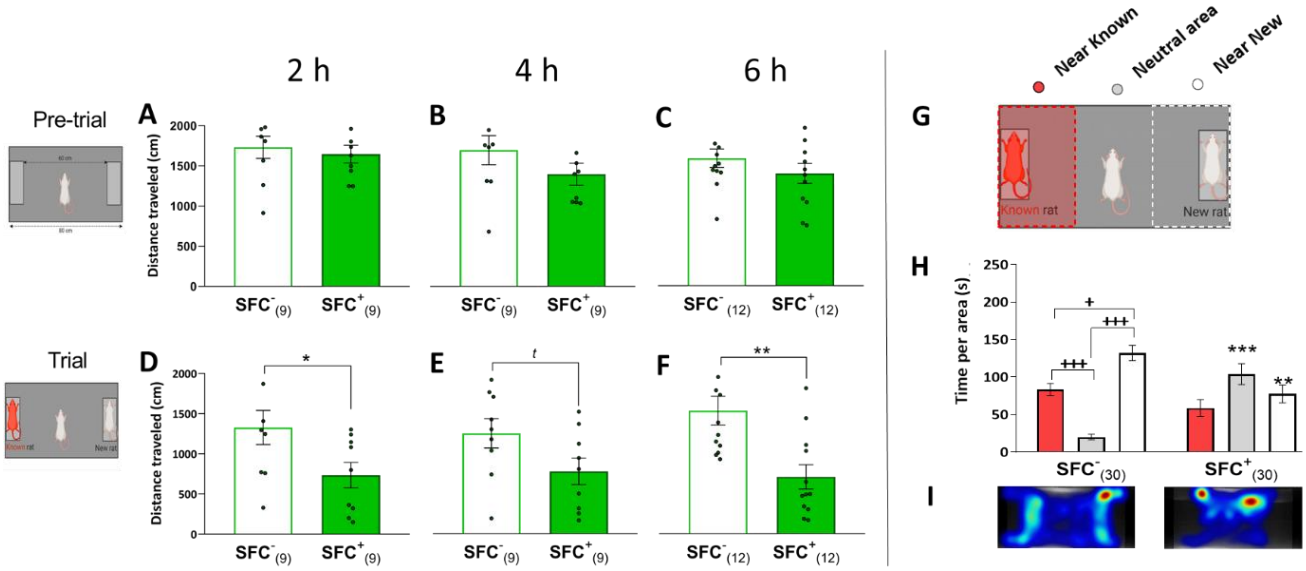


Fig.10 Locomotor activity of SFC⁻ and SFC⁺ rats. A-C) Pre-trial, and D-F) Trial, $^{*}p<0.05$, $^{**}p<0.01$, $^{t}p=0.073$. G) Analysis of the time spent in each area of the arena (equally divided into near the New stimulus (red color), neutral area (gray color) and the Known stimulus (white color). H) Average of the time spent in each area. $^{**}p<0.01$ and $^{***}p<0.001$, SFC⁻ *vs* SFC⁺, $^{+++}$ $p<0.001$ near know *vs* neutral *vs* near new areas. I) Representative heat maps of the arena exploration of SFC⁻ (left) and SFC⁺ (right) groups, a red color indicates longer time, while blue color indicates less time. All values represent the mean ±SEM.

Furthermore, I divided the arena into three equal areas: one near to the Known stimulus, one neutral area (center) and one near to the New stimulus (Fig. 10. G). Then, the software NOLDUS quantified the time that each animal spent in each area. Since there were no main effects of the retrieval interval (*i.e.*, no differences were found between 2 *vs* 4 *vs* 6 h within each SFC⁻ and SFC⁺), I pooled the data into SFC⁺ and SFC⁻ animals to gain more statistical power. A Two-way ANOVA for repeated measured indicated significant effects of SFC status (SFC⁺ *vs* SFC⁻) and Area (*i.e.*, near known *vs* neutral *vs* near new stimulus) (Table 9). Post-hoc analysis for SFC status revealed that the SFC⁺ group showed lower time spent near the new stimulus and higher time spent in the neutral area compared to the SFC⁻ group (Table 9, Fig. 10. H). Differences in time spent between areas were only observed in the SFC⁻ animals, which preferred to remain near to the new stimulus indicating social memory (Table 9, Fig.10.H).

Table 9. **Statistical analysis of the time spent per area (near Known, neutral or near New areas) in the social fear discrimination (two-way ANOVA of repeated measures).**

Factor (levels)	**Statistical**	***p* value**
SFC (SFC^+ vs SFC^-)	$F_{(1, 58)} = 5.39$	0.024^{*}
Area (near Known, neutral or New stimulus)	$F_{(1.962, 113.8)} = 6.32$	0.003^{**}
SFC X Area	$F_{(2, 116)} = 16.48$	$<0.0001^{***}$
Post hoc for SFC (Sidak test)	Near Known	0.219
SFC^- vs SFC^+	Neutral area	$<0.0001^{***}$
	Near New	0.003^{**}
Post hoc for Area (Tukey test)	SFC^-	SFC^+
Near Known *vs* Neutral area	$<0.001^{+++}$	0.124
Near Known *vs* Near New	0.029^{+}	0.581
Neutral area *vs* Near New	$<0.0001^{+++}$	0.503

3.1.3 Specificity of social fear conditioning

Toth and colleagues provided a control to exclude that shock itself and not pairing between the conspecific´s exploration and its punishment induces social fear (Toth et al., 2012b). They demonstrated the latter by giving five random shocks to a group of mice and comparing them with an unconditioned group (SFC^-, *i.e*., mice exposed to a conspecific without shocks). This comparison showed no significant differences between the analyzed groups, demonstrating abovementioned fine-tuning of the CS-US-pairing (Toth *et al.,* 2012).

Here, I extended this observation to rats, including the SFC^- and the shock groups, as well as two extra control groups (Fig.11.A, see a more detailed description of each group in *section 2.9.1*), namely the **unpaired group** (*i.e.,* each subject received four random shocks and unpaired with the presence of a conspecific, in other words, without punishing the social investigation) and the **non-social group** (*i.e.,* subject received four random shocks in presence of an empty cage). Then, I assessed how different CS-US pairings affect social fear discrimination within a retrieval interval of 6 h. No significant differences in social investigation time were observed between any groups (Fig.11.B; $F_{(3, 34)} = 2.52$; $p=0.074$). However, group comparisons showed a trend in the total freezing time (Fig.11.C, $H=7.63$; $p=0.054$). *Post hoc* analysis revealed that the unpaired group showed higher freezing levels compared to the non-social group ($p= 0.035$). in addition, none of the groups showed social discrimination (Data not shown).

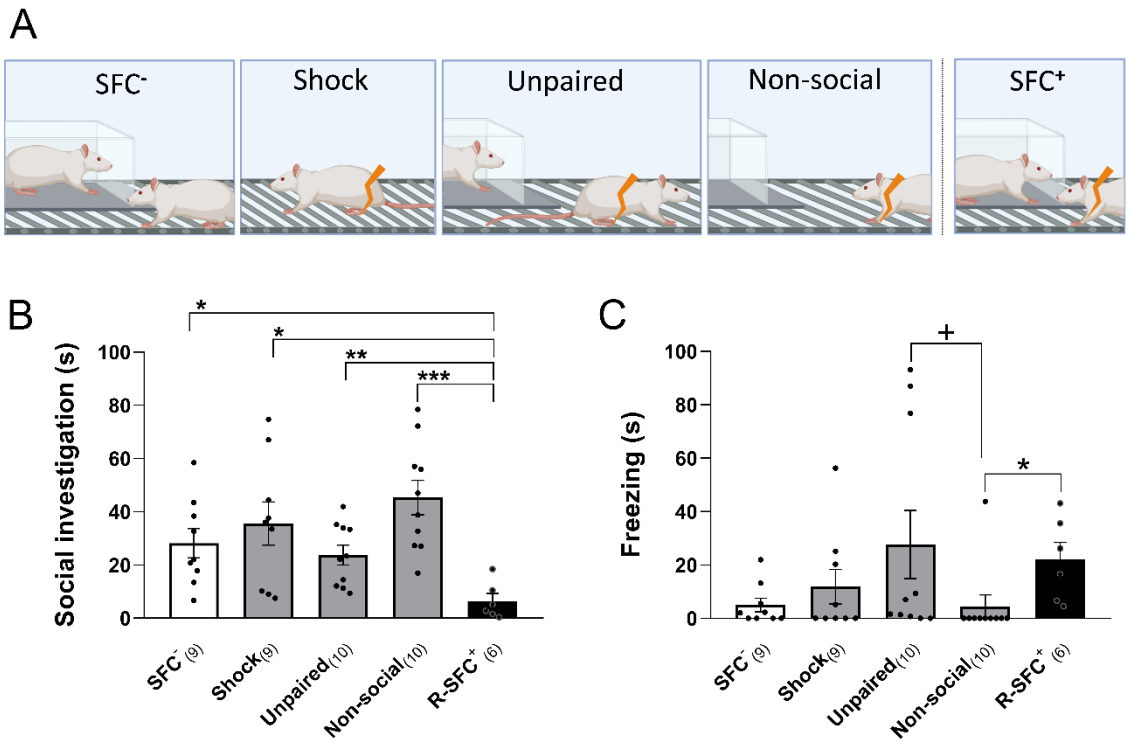


Fig.11. Effects of different CS-US pairings on social investigation and freezing during the social fear discrimination trial. A) Experimental groups: SFC$^-$ group did not receive any electric foot shocks. The remaining groups received four shocks, either randomly in the absence of a cage (shock group), randomly in the presence of a cage with a conspecific (unpaired group), randomly in the presence of an empty cage (non-social group), or during investigation of the conspecific (R-SFC$^+$). The R-SFC$^+$ group value was calculated from previous experiments to illustrate the specific pair response with four CS-US pairings. B) Social investigation time and C) Freezing time. ^+p=0.035 SFC$^-$ versus non-social group. *p<0.05, **p<0.01, ***p<0.001 each group versus R-SFC$^+$. All data are presented as mean ±SEM, sample size is given in parenthesis for each group.

In addition, to illustrate the effect of the specificity of the CS-US pairing, I included a **reference SFC$^+$** group (**R-SFC$^+$**), *i.e.*, subjects received a shock every time it explored the conspecific. This group was based on data of previous experiments of subjects that required similar CS-US pairings, *i.e.,* 4 ±0.6 shocks that were tested at the same retrieval interval of 6 h. The specificity of CS-US pairings was reflected by groups differences in social investigation (Fig. 11 B) and freezing times (Fig.11 C) (see *p* values in Table 10). *Post hoc* analysis revealed that the R-SFC$^+$ group showed reduced social investigation compared to each other group (see p values in Table 10). Differences in the time spent freezing were only observed between R-SFC$^+$ and the non-social group (Table 10).

Table. 10. Comparisons between CS-US nonspecific pairing groups and the R-SFC$^+$ group (Fig.11).

Group comparisons	**Statistical**	***p* value**
Social investigation (Welch´s ANOVA)	$F_{(4.00, 19.20)}$=10.25	0.0001****
Freezing time (Kruskal-Wallis test)	H=12.60	0.013*
Post hoc analysis	Social investigation (s)	Freezing (s)
(Dunnett´s test)	***p* value**	***p* value**
R-SFC$^+$ *vs* SFC$^-$	0.013*	0.175
R-SFC$^+$ *vs* Shock	0.021*	0.210
R-SFC$^+$ *vs* Unpaired	0.008**	>0.99
R-SFC$^+$ *vs* Non-social	<0.001***	0.006**

The final protocol can be found in the *section 2.6.3* with a detailed description of the procedure.

3.2 **Innate anxiety-related behavior influences susceptibility social fear conditioning**

Risk factors for SAD include BI to unfamiliar situations, genetic predisposition, and social trauma (Spence and Rapee, 2016). In my previous results, I showed that SFC induces social fear against same-sex conspecifics in rats up to 6 h, thus mimicking a social trauma experience. My following hypothesis was that combining SFC with other risk factors the social fear memory in rats may be prolonge. In this regard, the innate high anxiety-related behavior of HAB rats resemble the BI in SAD patients (*see 1.2.1*). Furthermore, the use of HAB and LAB rats, may facilitates the study of the genetic susceptibility to social fear (Landgraf and Wigger, 2002). Thus, I compare the SFC response between HAB, LAB, and NAB rats. NAB rats as control of any extreme trait in anxiety-related behavior.

3.2.1 Long-term social fear memory in HAB, NAB and LAB rats

First, all animals were tested in the EPM to confirm their anxiety levels at the age of 9 weeks. Three weeks later, they were tested in the social preference test (Lukas et al., 2011), as base line control of social behavior. After another week they were divided into conditioned (SFC^+, which received a shock, when exploring a conspecific) and unconditioned (SFC^-, free exploration) rats during social fear acquisition. Finally, social fear discrimination was performed after 24 h.

Elevated plus maze: As expected, the anxiety-related behavior level of HAB, LAB and NAB animals based on the percentage of time spent on the open arms differed (HAB: 7.26 ±5.06, NAB: 10.91 ±7.49, and LAB: 52.51 ±4.22). LAB rats spent significantly more time in the open arm compared to HAB and NAB rats (Table 11, Fig.12. A). No significant differences were observed in the latency to enter the open arm for the first time (Fig. 12.B). Besides, I summed up the entries in both arms and found the HAB rats displayed a lower number of entries compared to NAB and LAB rats (Fig. 12.C).

Social preference test: HAB, LAB, and NAB rats showed similar social investigation (Fig. 12.D; Table 11), and showed social preference behavior as assessed by the Preference ratio (*i.e.,* difference of time investigating the social *vs* the non-social stimulus divided by the total investigation time)(Fig. 12.E; Table 11).

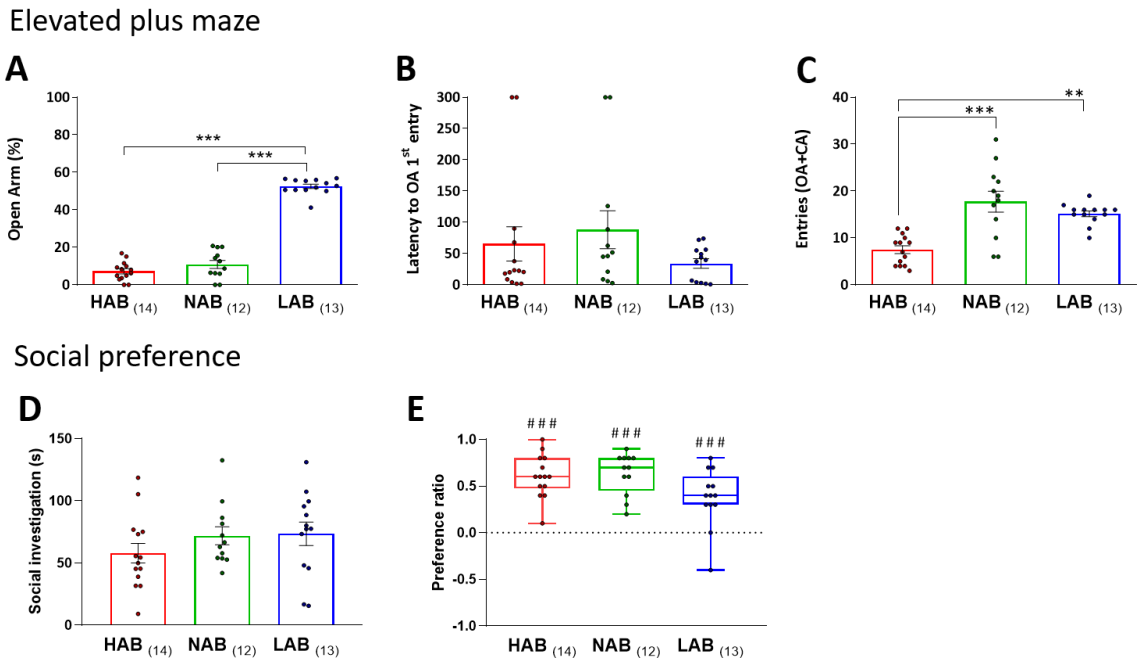


Fig. 12. Elevated plus-maze (EPM) and social preference behaviors in male rats selected for high (HAB) and low (LAB) anxiety-related behavior, and non-selected (NAB) rats. EPM response: A) time open arm in percentage, B) latency to enter the open arm (OA) for the first time and C) total entries. Between-group comparisons were indicated as $^{**}p<0.01$ and $^{***}p<0.001$. Social preference test: D) social investigation time and E) Preference ratio (Social time – Non-social time) / (Social time + Non-social time). One-sample T-test (control value zero) within each group is indicated with $^{###}$ $p<0.001$. All data represent the mean ± SEM.

Table 11. Statistical analysis of elevated plus maze (EPM) behaviors and social preference test in HAB, NAB and LAB rats (Fig.12).

Test	Variable	Factor (levels)	Statistical	*p* value
EPM	Time in open arm (%)	Line (HAB vs NAB vs LAB)	H=26.15	<0.0001***
	Latency to OA 1st entry	(Kruskal-Wallis test)	H=2.08	0.353
	Entries (OA+CA)		H=19.76	<0.0001***
	Post hoc (Dunn´s test)	Time in open arm (%)	Entries (OA+CA)	
	HAB vs. NAB	>0.999	0.0001***	
	HAB vs. LAB	<0.0001***	0.0014**	
	NAB vs. LAB	0.0004***	>0.999	
Social Preference	**Variable**	**Factor (levels)**	**Statistical**	***p* value**
	Social investigation (s) (One-way ANOVA)	Line (HAB vs NAB vs LAB)	$F_{(2, 36)}$ = 1.11	0.339
	Preference ratio (One sample T test)	HAB	t_{13}=9.86	<0.0001$^{###}$
		NAB	t_{11}=9.84	<0.0001$^{###}$
		LAB	t_{12}=4.33	0.001$^{###}$

Social fear acquisition

The three lines (NAB, LAB, HAB) showed different CS-US pairings, although a significant was not reach a strong trend was observed (Table 12). *Post hoc* test showed that NAB rats required significantly more electric foot shocks (4.11 ±2.14) than HAB rats (2.2 ±0.9) (Table 12). LAB rats (3.11 ±1.6) did not differ from the other groups (Table 12).

Table 12. Social fear conditioning analysis of HAB, NAB, and LAB rats (Fig. 13).

Test	Variable	Factor (levels)	Statistical	*p* value
SFC Acquisition	CS-US pairings (One-way ANOVA)	Line (SFC^+/HAB vs. SFC^+/NAB vs. SFC^+/LAB)	$F_{(2, 25)} = 3.31$	0.0531^t
	Post hoc: Line factor (Tukey´s test)	CS-US pairings	HAB vs NAB	0.042^*
			HAB vs LAB	0.4492
			NAB vs LAB	0.402
Social fear discrimination	Social investigation (s) (Two-way ANOVA)	Line	$F_{(1, 51)} = 7.07$	0.002^{**}
		SFC	$F_{(1, 51)} = 50.84$	$<0.0001^{***}$
		Line x SFC	$F_{(2, 51)} = 8.41$	0.001^{***}
	Freezing (s) (Two-way ANOVA)	Line	$F_{(1, 51)} = 5.60$	0.006^{**}
		SFC	$F_{(1, 51)} = 20.79$	$<0.0001^{***}$
		Line x SFC	$F_{(2, 51)} = 4.71$	0.013^*
	Post hoc: Line factor (Bonferroni´s test)	Social investigation (s)	HAB vs LAB	0.422
			HAB vs NAB	0.001^{***}
			LAB vs NAB	0.088
		Freezing (s)	HAB vs LAB	0.356
			HAB vs NAB	0.222
			LAB vs NAB	0.005^{**}

Social fear discrimination after 24 hours

I found a significant interaction between rat line and SFC (SFC^+ vs SFC^-) in the social investigation time, as well as in freezing time during the social fear discrimination trial performed 24 h after social fear acquisition (Table 12). *Post hoc* analysis showed a significant reduction in social investigation in the SFC^+ groups compared to respective SFC^- groups in HAB and LAB rats, but not in NAB rats (Table 12. Fig. 13 A-C). In contrast, the freezing time was increased in SFC^+ compared to SFC^- rats in all three lines (Table 13). Altogether, these results reflect an increased susceptibility of animals with an extreme trait in anxiety-like behaviors (*i.e.*, HAB and LAB) to acquire social fear, whereas NAB rats seem to be rather resilient. Additionally, only the SFC^+ HAB rats were capable to discriminate the known stimulus, indicating individual social fear memory (Fig. 13.G, Table 13).

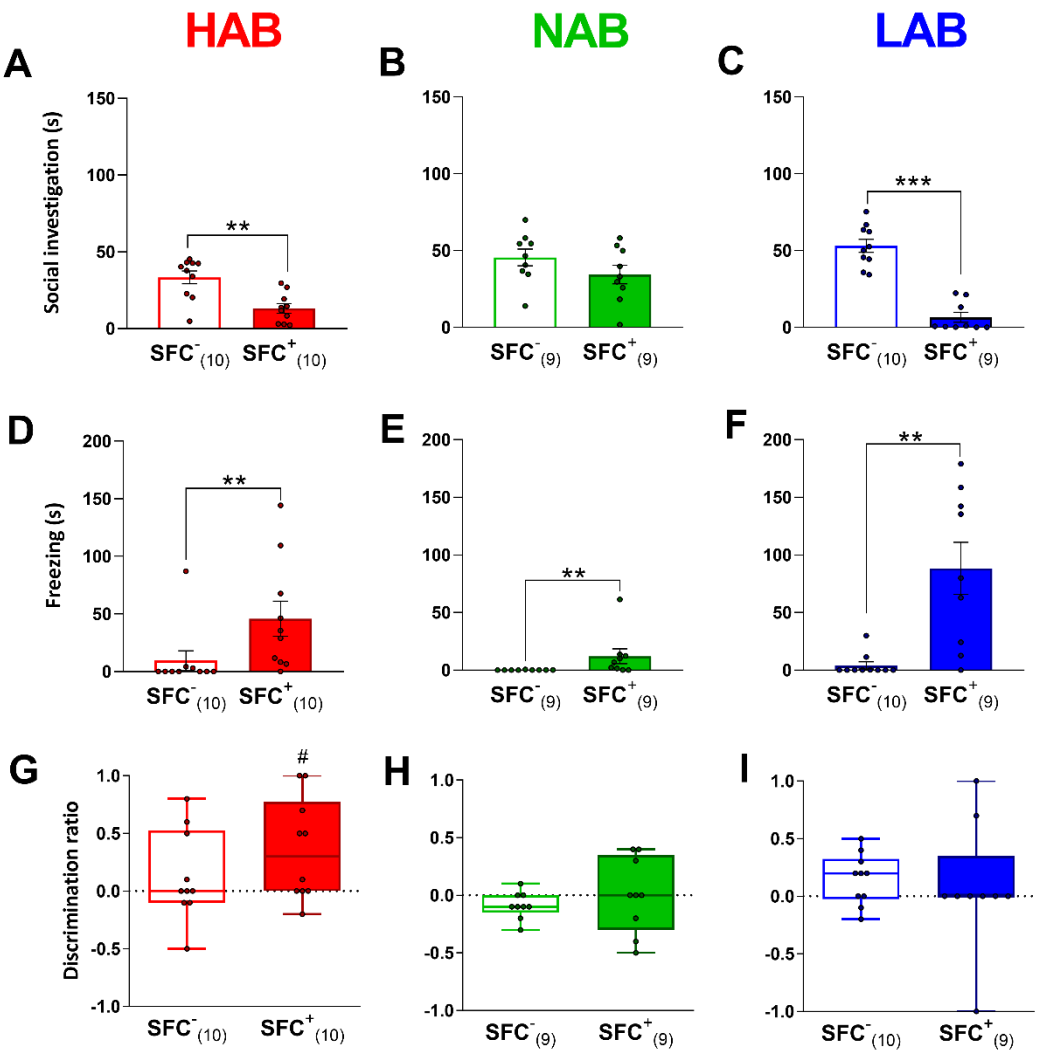


Fig. 13. Behavioral responses during the social fear discrimination trial in male rats selectively bred for high (HAB) and low (LAB) anxiety-related behavior, and non-selected (NAB) rats. A-C) Social investigation time, D-F) Freezing time, G-I) Discrimination ratio. $^{**}p<0.01$ and $^{***}p<0.001$, indicate between group comparisons. $^{\#}p<0.05$ indicates significant differences from zero in a one sample t-test. Data represent mean ± SEM, group sizes are given in parentheses.

Finally, correlation analysis was performed to assess, whether EPM parameters, social investigation during the social preference test or the number of CS-US pairings during social fear acquisition, were correlated to social fear parameters in the social fear discrimination trial. However, there was no significant correlation between the above-mentioned variables, except for a significant negative correlation between social investigation and freezing time both measured during the social fear discrimination trial (Table 14).

Table 13. *Post hoc* analysis of the interaction SFC x Line in HAB, NAB, and LAB rats.

Variable	SFC⁻ *vs* SFC⁺	Statistical	*p* value
Social investigation (Unpaired T test)	HAB	t_{18}=3,88	0.001***
	NAB	t_{16}=1.36	0.191
	LAB	t_{17}=8.52	<0.0001***
Freezing time (Mann-Whitney test)	HAB	*U*=13.5	0.003**
	NAB	*U*=10	0.002**
	LAB	*U*=8.5	0.001***
Discrimination ratio (One-sample T-test)		**SFC⁻**	**SFC⁺**
	HAB	*p*=0.318	*p*=0.029*
	NAB	*p*=0.052	*p*>0.999
	LAB	*p*=0.062	*p*=0.683

Table 14. Spearman´s rho correlation analysis between variables measured in the elevated plus maze (EPM), social preference test and social fear parameters in conditioned HAB, LAB and NAB rats. N=19

Test	Variable	Statistical *p* value	CS-US	Basal social investigation	Social investigation	Freezing (s)
EPM	**Open arm (s)**	r_s	0.210	0.290	-0.413	0.423
		p	0.387	0.229	0.079	0.071
	Closed arm (s)	r_s	-0.186	-0.186	0.349	-0.331
		p	0.447	0.446	0.143	0.166
	Open arm (%)	r_s	0.232	0.221	-0.369	0.392
		p	0.340	0.363	0.120	0.097
SFC acquisition	**CS-US pairings**	r_s		0.046	0.050	0.194
		p		0.853	0.801	0.322
Social Preference	**Basal social investigation**	r_s			-0.321	0.132
		p			0.180	0.591
SFC- Social fear discrimination	**Social investigation**	r_s				-0.649
		p				<0.001**

Based on the previous results, *i.e*., the long-term social fear memory of HAB and LAB, but not NAB rats, I tested, whether the observed differences were related to differences in their learning abilities or pain sensitivity. Therefore, I used a learning paradigm similar to the SFC paradigm, but in a non-social context, *i.e*. cued fear conditioning, which I performed 2 weeks after SFC. Moreover, animals were tested in the Hargreaves' plantar test 3 weeks after SFC to control for pain sensitivity.

Cued fear conditioning

Some animals were excluded from this analysis due to health issues, and data of some subjects is missing for the extinction training due to a mechanical problem with the conditioning chamber during the extinction phase (*i.e*., some animals were not properly detected by the sensor beans). To keep statistical power, I did not conduct the traditional repeated measures analysis (*i.e*., compare along five time the freezing response between the groups), instead, I selected the freezing (%) at the fifth CS-US pair as a general indicator of fear learning between the groups during fear acquisition. For fear extinction analysis, I calculated the reduction of freezing time, as the difference between the last and the first CS presentations. Thus, a negative value indicates extinction or freezing loss, while positive values indicated increasing freezing levels and impaired extinction.

During cued fear acquisition, no rat line specific significant effects on freezing levels were observed (Table 15). Nevertheless, the freezing levels were generally higher in HAB, LAB, and NAB groups that previously experienced SFC^+ compared with SFC^- groups (Fig. 14. A, Table 15). This effect reached statistical significance in the *post hoc* analysis in HAB rats (p=0.002). During fear extinction on the next day, I found a significant difference between the three rat lines (Table 15), with a successfully extinction only in LAB and NAB rats. In contrast, SFC^-/HAB rats showed significant higher freezing levels than SFC^-/LAB and SFC^-/NAB rats (indicated by negative values). Further, SFC^+/HAB showed higher freezing levels than SFC^+/LAB (Fig. 14. B). During recall on day 3, there was a significant effect of SFC, all SFC^+ groups showed higher freezing than SFC^- groups (Data no shown). Moreover, neither line nor interaction effects were found (Table 15).

Pain sensitivity

My previous results showed that HAB rats required less electric foot shocks than NAB rats during social fear acquisition. As this could be related to either a higher sensitivity or perception of the pain between the lines. I evaluated the pain sensitivity between HAB, LAB, and NAB male rats with the **Hargreaves' plantar test** (**HT**). Since I did not observe an effect of habituation (*i.e*., differences between replicas, since each paw was measured three times), or any asymmetrical response (*i.e.,* left *vs* right paw measures) (data no shown), I calculated an average of the paw withdrawal latency per animal. Analysis did not reveal differences between lines (Table 14; Fig.15). Nevertheless, a significant effect of previous exposure to SFC was found (Table 15). No interactions were observed between Line x SFC (Table 15). *Post hoc* analysis showed a trend in HAB/SFC^+ rats to higher pain

sensitivity reflected by faster reaction to the thermal stimulation than the HAB/SFC$^-$ (Fig.14; t_9=2.17; p= 0.058).

Table 15. Statistical analysis for cued fear conditioning and and Hargreaves' plantar test (HT).

Test	Variable	Factor (levels)	Statistical	*p* value
CFC	Acquisition	Line	$F_{(2, 30)}$ = 0.95	0.397
	5th CS-US freezing (%)	SFC	$F_{(1, 30)}$ = 11.62	0.002^{++}
	(Two-way ANOVA)	Line x SFC	$F_{(2, 30)}$ = 2.67	0.086
	Extinction	Line	$F_{(2, 20)}$ = 24.04	<0.001***
	Freezing reduction (%)	SFC	$F_{(1, 20)}$ = 3.80	0.065
	(Two-way ANOVA)	Line x SFC	$F_{(2, 20)}$ = 2.09	0.148
	Post hoc for line effects		SFC$^-$	SFC$^+$
	(Bonferroni´s test)	HAB vs NAB	0.002**	0.052
		HAB vs LAB	<0.0001****	0.010*
		NAB vs LAB	0.108	>0.999
	Recall	Line	F $_{(2, 30)}$ = 1.69	0.201
	Freezing (%)	SFC	F $_{(1, 30)}$ = 6.07	0.019^{+}
	(Two-way ANOVA)	Line x SFC	F $_{(2, 30)}$ = 0.16	0.850
HT	Paw withdrawal latency (s)	Line	F $_{(2, 30)}$ = 1.48	0.243
	(Two-way ANOVA)	SFC	F $_{(1, 30)}$ = 8.60	0.006^{++}
		Line x SFC	F $_{(2, 30)}$ = 0.51	0.604

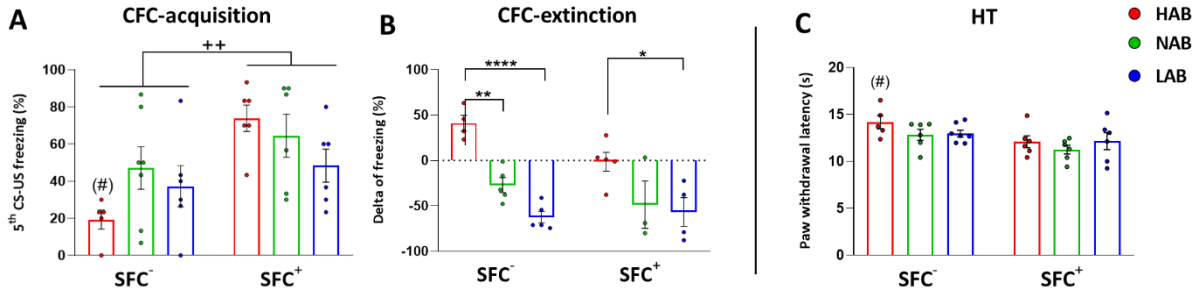


Fig.14 Cued fear conditioning (CFC) and Hargreaves' plantar test (HT) responses in male rats bred for high (HAB) and low (LAB) anxiety-related behavior, and non-selected (NAB) rats. A) Cued fear acquisition indicated by the freezing levels observed in the 5th CS-US pair (sample size 5-7 rats per group),^{++}p<0.01 main effects for SFC$^-$ vs. SFC$^+$. **B)** Freezing reduction after the extinction training (calculated as the difference between the last - first CS presentation). Sample size 3-6 rats per group, *p<0.05, **p<0.001, ****p<0.0001 differences between groups. C) Paw withdrawal latency in the HT (sample size 5-7 rats per group), $^{\#}p$=0.05: HAB/SFC$^-$ *vs.* HAB/SFC$^+$. All data represent mean ±SEM.

3.3 Involvement of vasopressin in long-term social fear memory in rats

It was previously shown, that HAB rats carried an SNP in the AVP gene promoter causing an *in vivo* overexpression of AVP (Murgatroyd et al., 2004). As well, previous findings showed that AVP acts an enhancer of the social recognition (*see section 1.3.3*). This suggests a mechanism that mediates the long-term social discrimination of the Known *vs* New stimulus in conditioned HAB rats described above (Fig.13 G). Therefore, to test the hypothesis that AVP contributes the individual recognition and eventually the social fear after SFC, I infused animals either centrally (icv) or locally (lateral septum) with either vehicle (Ringer solution), synthetic AVP or an AVP receptor antagonist (V1a-A) immediately after the social fear acquisition and evaluated the effects 24 h after the social fear discrimination test. The results are described in the following sections.

3.3.1 Central vasopressin effects on SFC consolidation

For my first approach I centrally manipulate the AVP system, rats were implanted with a guide cannula placed 2 mm above the lateral ventricles. After three days of recovery, rats were subjected to SFC and split in SFC^+ and SFC^- groups, followed by the respective icv infusion. HAB rats, received either **AVP** (1ng/5µl), AVP receptor antagonist **(V1a-A**, 10ng/) or Ringer solution (**vehicle group: Veh**). LAB rats, which showed social fear without social discrimination, received the same treatments, , but an additional group was infused with a low dose of AVP (**AVP-L,** 0.5 ng/5µl). NAB rats did not show social fear nor social discrimination, received either a high dose of AVP (1ng/5µl) or Veh .

Social fear acquisiton

In confirmation of the results above, there were significant differences between lines in the number of CS-US pairings (number electric foot shocks, *KW*= 18.15, *p*=0.0001; Fig.15 A). NAB rats required significant more CS-US pairings (*p* <0.0001 *vs* HAB; *p*=0.065 *vs* LAB). In addition, within each line, there were no differences between SFC^+ groups that later were split in different drug treatments (Fig. 15. B-D, Table 16), this suggest a similar acquisition and control for the specificity of effects due the treatment.

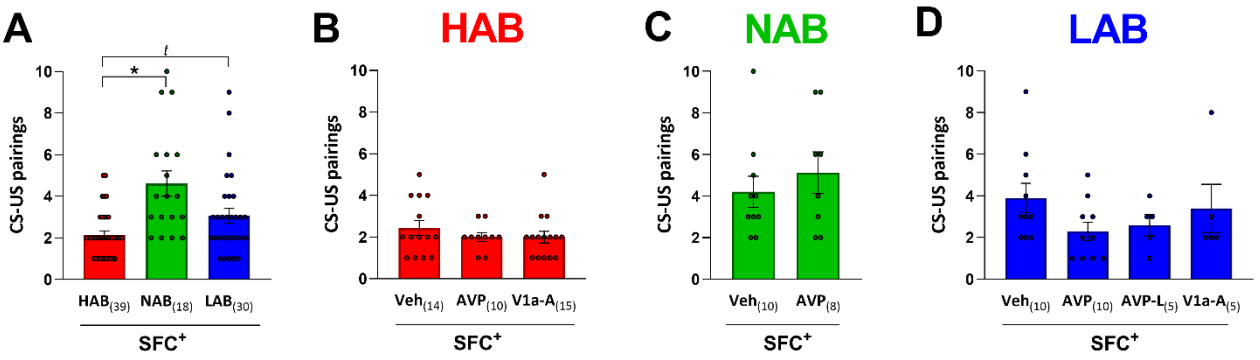


Fig. 15. CS-US pairings (number of electric foot shock) during social fear acquisition in high (HAB) and low (LAB) anxiety-related behavior and non-selected (NAB) rats. A) Comparison between rat lines acquisition. $^{*}p<0.05$, $^{t}p=0.065$. B) HAB rats acquisition. C) NAB rats acquisition and D) LAB rats acquisition. Data represent mean ± SEM, group sizes are given in parentheses.

Table 16. Statistical analysis of the social fear conditioning parameters in high (HAB) and low (LAB) anxiety-related behavior and non-selected (NAB) rats.

Behaviors	HAB	NAB	LAB
CS-US pairings (Kruskal-Wallis and U Mann Whitney test)	*H*= 0.73 *p*=0.694	*U*=33.50 *p*=0.572	*H*= 4.0 *p*=0.261
Social investigation (Kruskal-Wallis test)	*H*=33.17 *p*<0.0001****	*H*=4.14 *p*=0.247	*H*=25.13 *p*=0.0001***
Post hoc			
Social investigation (Dunn's test)	SFC⁻/Veh vs. SFC⁺/Veh: *p*=0.001*** SFC⁻/Veh vs. SFC⁺/AVP: *p*=0.003** SFC⁻/Veh vs. SFC⁺/V1a-A: *p*=0.173 SFC⁺/Veh vs. SFC⁺/V1a-A: *p*=0.046(+)		SFC⁻/Veh vs. SFC⁺/Veh: *p*=0.0003** SFC⁻/Veh vs. SFC⁺/AVP: *p*=0.339 SFC⁻/Veh vs. SFC⁺/AVP-L: *p*=0.268 SFC⁻/Veh vs. SFC⁺/V1a-A: *p*=0.011* SFC⁺/Veh vs. SFC⁺/ AVP: *p*=0.149
Freezing time (Kruskal-Wallis test)	*H*=24.84 *p*<0.0001****	*H*=9.41 *p*=0.024*	*H*=24.19 *p*=0.0002***
Post hoc			
Freezing time (Dunn's test)	SFC⁻/Veh vs. SFC⁺/Veh: *p*=0.098 SFC⁻/Veh vs. SFC⁺/AVP: *p*=0.003** SFC⁻/Veh vs. SFC⁺/V1a-A: *p*>0.999 SFC⁺/Veh vs. SFC⁺/ V1a-A: *p*=0.551	SFC⁻/Veh vs. SFC⁺/Veh: *p*=0.044* SFC⁻/Veh vs. SFC⁻/AVP: *p*>0.999 SFC⁻/Veh vs. SFC⁺/AVP: *p*>0.999 SFC⁺/Veh vs. SFC⁺/ AVP: *p*=0.453	SFC⁻/Veh vs. SFC⁺/Veh: *p*=0.011* SFC⁻/Veh vs. SFC⁺/AVP: *p*>0.999 SFC⁻/Veh vs. SFC⁺/AVP-L: *p*>0.999 SFC⁻/Veh vs. SFC⁺/V1a-A: *p*=0.038* SFC⁺/Veh vs. SFC⁺/ AVP-L: *p*=0.033*
Discrimination ratio (One sample T test)	SFC⁺/Veh: *p*= 0.031#	SFC⁻/AVP: *p*=0.039*	none

Social fear discrimination

Central blockage of V1a receptors prevented individual social fear memory consolidation in conditioned HAB rats

During the social fear discrimination trial, I found significant differences between HAB treatment groups in the social investigation and freezing time (Table 16). SFC^+ HAB rats treated with Veh or AVP showed a significant reduction in the total social investigation time compared with SFC^-/Veh group (Fig. 16 A; Table 16). In contrast, SFC^+/ HAB rats treated with the AVP antagonist (SFC^+/V1a-A) did not differ from SFC^-/Veh, but instead showed higher social investigation compared to SFC^+/Veh rats (Fig.16 A; Table 16). These results suggest that the AVP-antagonist prevented the consolidation of social fear memory. Analysis of the freezing time showed differences only between the SFC^-/ Veh and SFC^+/AVP group, where the latter group showed the higher freezing levels (Fig. 16 B). As expected, the SFC^+/Veh group displayed social discrimination, however, neither synthetic AVP nor the AVP antagonists altered social discrimination (Fig. 16 C; Table 16).

Central AVP infusion prevented social fear memory consolidation in conditioned LAB rats

During the social fear discrimination trial in LAB rast, I found significant differences between treatment groups in the total social investigation and freezing time (Table 16). Both, SFC^+/Veh and SFC^+/V1a-A, showed reduced social investigation compared to SFC^-/Veh (Fig. 16 D). Contrary, SFC^+ groups treated with high and low doses of AVP did not differ from the SFC^-/Veh group indicating reduced social fear (Table 16). However, *post-hoc* analysis showed that only the SFC^+/AVP-L group significantly differed from the SFC^+/Veh group (Table 16). Further, *post-hoc* analysis of the freezing time showed that the SFC^+/Veh and SFC^+/V1a-A group displayed significantly higher levels of freezing compared to SFC^-/Veh (Fig. 16 E, Table 16). In contrast, SFC^+ groups of high and low AVP doses showed reduced freezing in general, but significant drug effects were only observed between SFC^+/AVP-L and SFC^+/Veh (Table 16). Finally, none of the groups showed social discrimination (Fig. 16 F, statistics not shown).

Central AVP infusions did not affect social fear in conditioned NAB rats but prolonged social discrimination of unconditioned NAB rats up to 24 hours.

No significant differences were observed in the social investigation time between the NAB treatment groups (Fig.16 G, Table 16). This is in line with previous results, where NAB rats did not express long-term social fear memory (24 h). However, I found differences between the groups in

the freezing time (Table 16). *Post hoc* analysis indicated that the SFC$^+$/Veh rats showed higher freezing time compared to SFC$^-$/Veh (Fig.16 H, Table 16). Notably, only the SFC$^-$/AVP group showed social discrimination after 24 h (Fig.16 I, Table 16).

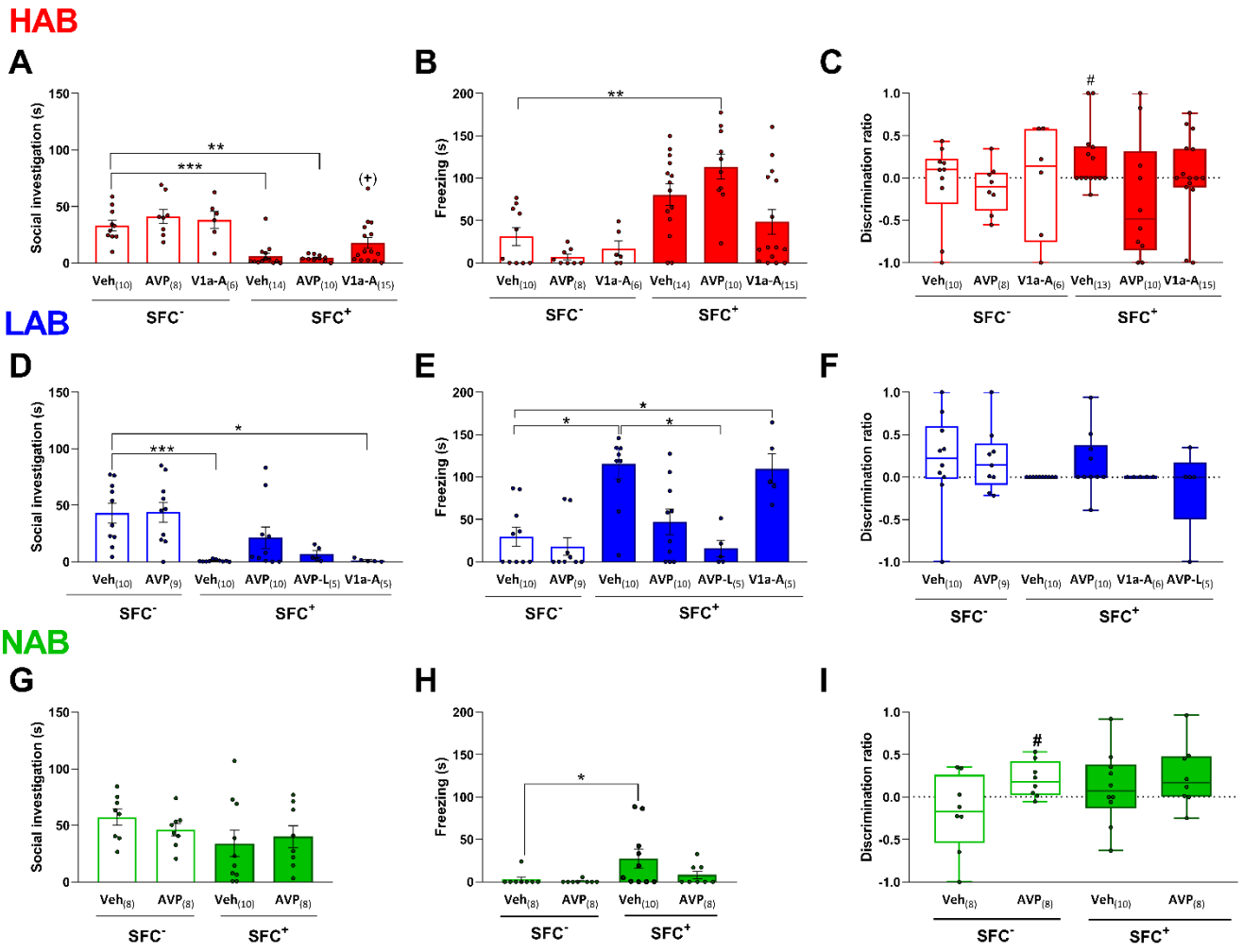


Fig.16. Social fear discrimination trial response in high (HAB) and low (LAB) anxiety-related behavior and non-selected (NAB) rats. A, D, G) Social investigation. **B, E, H)** Freezing time. **C, F, I)** Discrimination ratio (positive values indicated preference for the New stimulus). *p<0.05, **p=<0.01, ***p<0.001, all indicated comparisons between groups. #p<0.05, indicated significant difference from zero in the One sample t-test. Data represent mean ± SEM, group sizes are given in parentheses.

3.3.2 Role of AVP in the lateral septum during SFC consolidation

Considerable evidence indicates that social recognition is regulated by the LS, and this in turn is mediated by the V1a receptor (*see 1.3.3*). Moreover, the LS is also involved in social fear extinction in mice, but mainly due to OXT regulation (Menon et al., 2018; Zoicas et al., 2014). Therefore, to investigate the potential role of AVP within the LS in social fear consolidation, as a follow up from the previous experiment, rats were implanted with a guide cannula above the LS (*see 2.2.1*). After four days of recovery, animals underwent social fear acquisition and were immediately thereafter bilaterally infused with either AVP, V1a-A, or Ringer solution into the LS (doses in*s 2.3*). After 24 h, the social fear discrimination was assessed. Anxiety-related phenotypes were confirmed for each line using EPM (9 weeks old), as well as an intact social preference using SPM as baseline controls (data no shown).

Social fear acquisition

HAB rats required less CS-US pairings than LAB rats (U= 82, p=0.029; Fig. 17 A). However, within each line there was no significant difference between the later drug treatments groups (Table 17), neither in HAB rats (Fig.17 B) nor LAB rats (Fig.17 C).

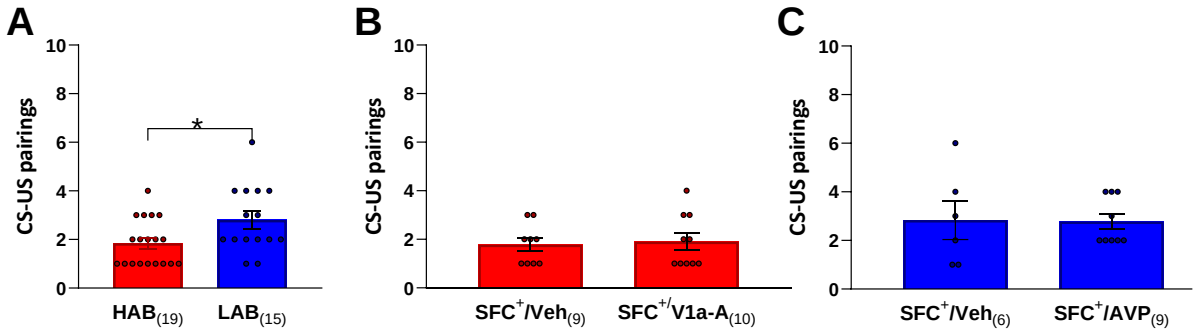


Fig. 17. CS-US pairings (number of electric foot shocks) during social fear acquisition in high (HAB) and low (LAB) anxiety-related behavior rats. A) Comparison between rat lines acquisition, $^{*}p$<0.05. B) HAB rats acquisition. C) LAB rats acquisition. Data represent mean ± SEM, group sizes are given in parentheses.

Social fear discrimination

Both SFC^{+} HAB and LAB groups showed a significant reduction of the total social investigation time (Fig. 18 A and D) accompanied by higher levels of freezing (Fig. 18 B and E) compared to their SFC^{-} /Veh groups, respectively (Table 17). Moreover, I did not find any differences between the vehicle groups and each drug treatment (Table 17), namely V1a-A in HAB rats or AVP in LAB rats. Finally, none of the groups showed social discrimination (Fig. 18 C and F, statistics no shown).

Table 17. Statistical analysis of the social fear conditioning parameters in high (HAB) and low (LAB) anxiety-related behavior and non-selected (NAB) rats. (Fig. 17 and 18)

Behaviors	HAB	LAB
CS-US pairings	*U*=44; *p*>0.999	*U*=24. 50; *p*=0.783
Social investigation (Kruskall-Wallis test)	*H*=14.86 *p*<0.0006***	*H*=25.13 *p*=0.0004***
Post hoc		
Social investigation (Dunn's test)	SFC-/Veh vs. SFC+/Veh: p=0.003** SFC-/Veh vs. SFC+/V1a-A: p=0.0011** SFC+/Veh vs. SFC+/V1a-A: *p*>0.999	SFC-/Veh vs. SFC+/Veh: p=0.052t SFC-/Veh vs. SFC+/AVP: p=0.002** SFC-/Veh vs. SFC+/AVP: p>0.999
Freezing time (Kruskall-Wallis test)	*H*=10.89 *p*<0.004**	KW=12.90 *p*=0.0002***
Post hoc		
Freezing time (Dunn's test)	SFC-/Veh vs. SFC+/Veh: p=0.009** SFC-/Veh vs. SFC+/V1a-A: p=0.008** SFC+/Veh vs. SFC+/V1a-A: *p*>0.999	SFC-/Veh vs. SFC+/Veh: p=0.030* SFC-/Veh vs. SFC+/AVP: p=0.001** SFC-/Veh vs. SFC+/AVP: p>0.999

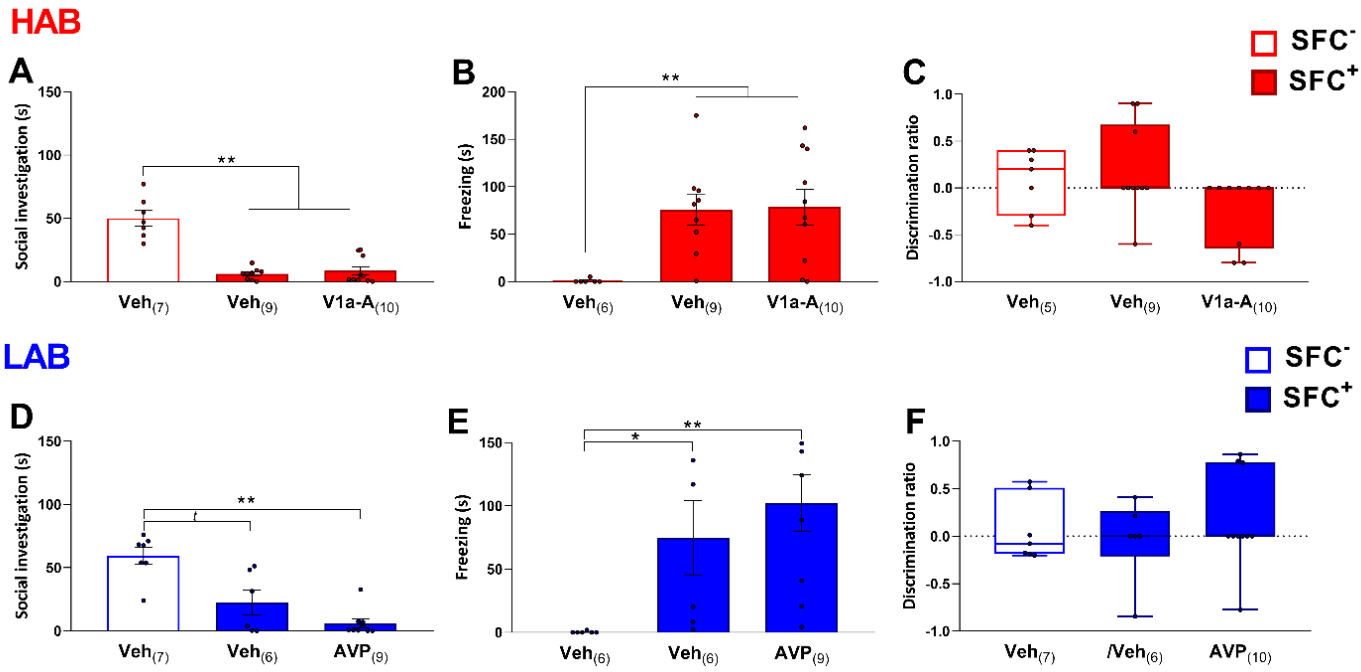


Fig.18. Social fear discrimination trial response in high (HAB) and low (LAB) anxiety-related behavior rats. A and D) Social investigation. B and E) Freezing time. C and F) Discrimination ratio (positive values indicated preference for the New stimulus). *p<0.05 and **p=<0.01 indicated comparisons between groups. Data represent mean ± SEM, group sizes are given in parentheses.

3.4 Glucocorticoids effects in social fear memory

Depending on the concentration and the temporal manner of administration GCs either enhance or impair memory processing (de Quervain et al., 2017b; Sapolsky et al., 2000). In the following experiments, I investigated the impact of GCs on the long-term consolidation of social fear memory in HAB and LAB rats, since both lines showed robust social fear memory after 24 h (*i.e.* reduced social investigation and higher freezing, although only HAB rats showed individual discrimination). Interestingly, both lines showed a dysregulation of the stress response (*see 1.4.2*). First, I compared the plasma Cort levels between (HAB *vs* NAB) and within lines (SFC^+ *vs* SFC^-) during the different phases of SFC. LAB rats were excluded from this experiment due to limited reproductive rates. Based on the higher basal Cort levels in HAB compared to NAB rats, my second approach was to assess the effect of Cort signaling blocking in HAB and LAB rats before the acquisition of social fear on memory consolidation. Finally, I assessed the effects of Cort treatment (ip or icv) in NAB rats, to test if higher Cort level in NAB rats prolong the social fear memory from 6 to 24 h.

3.4.1 Cort release during SFC in HAB and NAB

To measure the plasma Cort release, rats were implanted with a JVC. Four days post-surgery, they were tested in the SFC. One hour after attaching the lines to the rat's catheter, a basal blood sample was collected. Afterwards, blood samples were collected during the conditioning at 5, 15, and 60 min following shock exposure in the SFC^+ group or following the first social approach in the SFC^- group. Social fear discrimination was evaluated circa 6 h after the conditioning. Two more blood samples were collected, a second basal sample (one hour before the social fear discrimination), and one 15 min after the presentation of the social stimuli.

Cort levels during SFC

Basal levels of Cort showed significant differences between rat lines (HAB *vs* NAB rats) and within lines (future SFC groups) (Table 18), where in general HAB rats showed higher Cort levels compared to NAB rats (Fig.19 A). In contrast, during the social fear acquisition no significant differences were observed between (HAB *vs* NAB) or within lines (SFC^+ *vs* SFC^-) (Table 18). However, a significant effect of time was observed (Table 18), *post hoc* tests revealed a significant rise of Cort levels 5 min after the first social approach compared to the basal levels (Fig. 19 A), this increase trend were maintained up to 15 min, and Cort levels returned to basal levels after 1 h (Table18). Similarly, during social fear discrimination, only a significant effect of time were observed between the groups (Table

18). In specific a significant rise of Cort was observed 15 min after the first social approach compared with the second basal level taken one hour prior the test (Fig. 19 B; Table 18). Notably, *post hoc* analysis showed that this increment was significant only in the SFC$^+$ groups (Table 18).

Social fear acquisition and social fear discrimination

HAB and NAB rats showed similar CS-US pairings (U=22, p= 0.3453; Fig.19 C). After 6 h, both groups showed social fear, indicated by a significant reduced social investigation (Fig.19 D, Table 19). However, a significant increase in freezing levels were observed only in HAB rats (Fig. 19 E, Table 19). Finally, both HAB groups discriminate between the Known and New stimuli, as well as the NAB/SFC$^-$ (Fig.19 F).

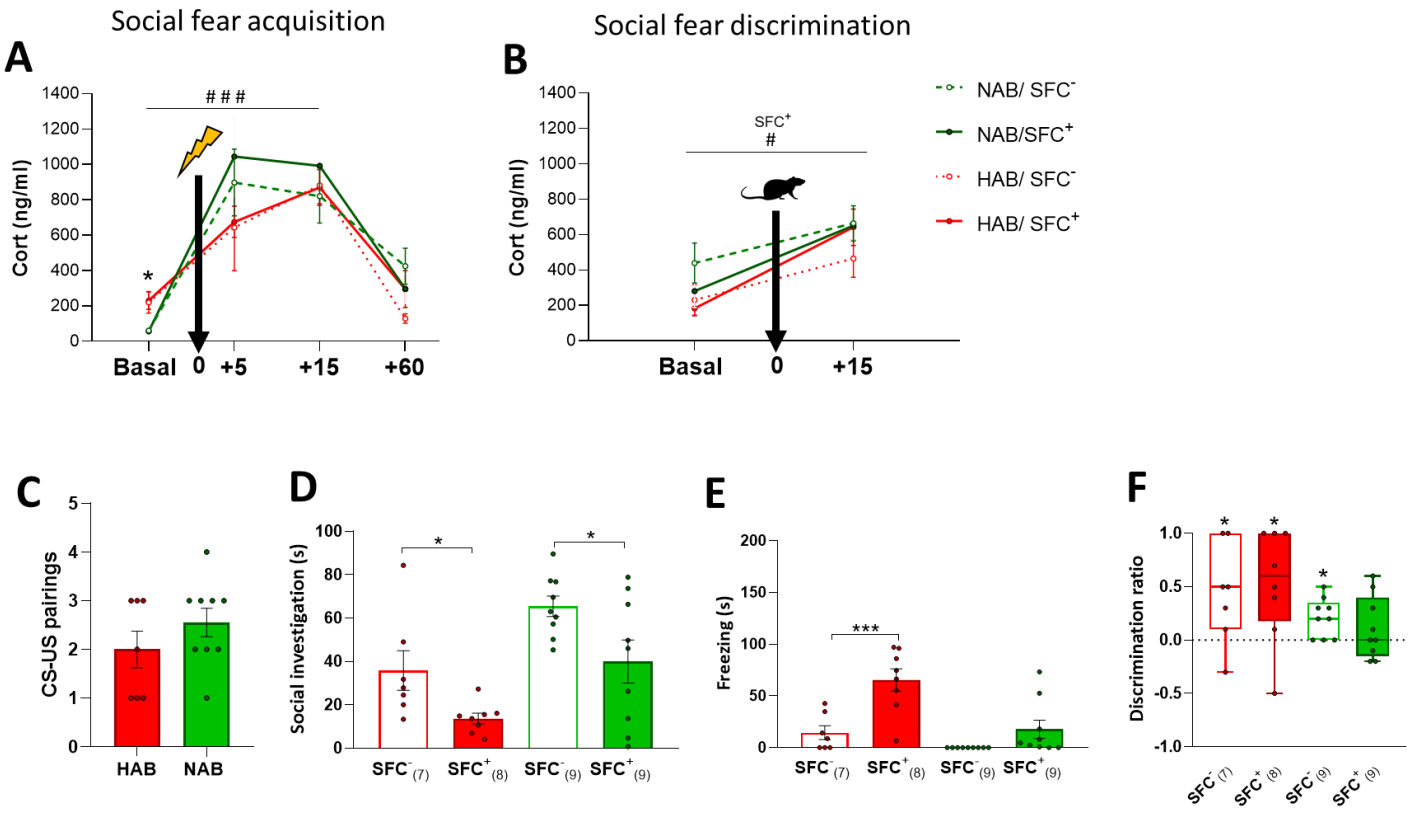


Fig. 19. Cort levels (mean ±SEM) and behavioral response in high anxiety-related (HAB) rats and non-selected (NAB) rats during SFC. A) Cort release during acquisition (the basal sample was collected after 1 h of habituation to the line, then each animal was conditioned and samples were collected at 5, 15, and 60 min after the first social approach in the conditioning chamber). B) Cort levels before and after social fear discrimination (basal samples were collected 1 h prior to testing and the second 15 min following the presentation to a social stimul. C) CS-US pairings during social fear acquisition. D) Social investigation time. E) Freezing levels. F) Discrimination ratio. Data represent mean ± SEM, group sizes are given in parentheses. *p<0.05: indicates comparisons between groups, but in F) indicates differences from zero value; #p<0.05 and ###p<0.0001 comparisons between time points.

Table 18. Analysis for Cort levels between HAB and NAB.

Test	Factor (levels)	Statistical	*p* value
Basal levels	Line (HAB vs NAB)	*U*=52	0.002**
	SFC (SFC groups)	*H*=6.71	0.0008**
	Post hoc Dunn's test	HAB/SFC$^-$ vs NAB/SFC$^-$	0.025*
		HAB/SFC$^+$ vs NAB/SFC$^+$	0.009**
		HAB/SFC$^-$ vs HAB/SFC$^+$	0.999
		NAB/SFC$^-$ vs NAB/SFC$^+$	0.999
Acquisition	Time (basal vs +5 vs +15 vs +60)	$F_{(2.072, 38.68)}$ = 37.18	0.0001****
	SFC (SFC groups)	$F_{(3, 29)}$ = 0.64	0.590
	Time x SFC	$F_{(9, 56)}$ = 1.295	0.260
	P*ost hoc* Bonferroni´s test	Basal vs +5	0.0001****
		Basal vs +15	0.0001****
		Basal vs +60	0.062
		+5 vs +15	0.999
		+5 vs +60	0.0001****
		+15 vs +60	0.0001****
Social fear discrimination	Time (basal vs +5 vs +15 vs +60)	$F_{(1, 56)}$ = 22.45	0.0001****
	SFC (SFC groups)	$F_{(3, 56)}$ = 1.62	0.193
	Time x SFC	$F_{(3, 56)}$ = 0.67	0.568
	P*ost hoc* Bonferroni´s test	Basal *vs* +15	
		NAB/ SFC$^-$ $_{(9)}$	0.330
		NAB/SFC$^+$ $_{(9)}$	0.026*
		HAB/ SFC$^-$ $_{(7)}$	0.441
		HAB/ SFC$^+$$_{(8)}$	0.007**

Table 19. Analysis of the behaviors during the social fear discrimination in HAB and NAB rats. (Fig.19)

	HAB	NAB
Social investigation	t_{13}=2.49	t_{16}=2,313
(Unpaired T test)	*p*= 0.026	*p*=0.034
Freezing time	*U*=5	*U=9*
(Mann-Whitney test)	*p*=0.005	*p*=0.002
Discrimination ratio **(One sample T test)**		
SFC$^-$	t_6=2.50; *p*=0.046*	t_8=3.45; *p*=0.008**
SFC$^+$	t_7=2.81; *p*=0.026*	t_8=1.13; *p*=0.288

3.4.2 Blocking of Cort synthesis before social fear acquisition impaired social fear consolidation in HAB and LAB rats

To evaluate the effects of reduced levels of Cort before the social fear acquisition, 90 min before social fear acquisition I injected (*ip*) HAB and LAB rats either with vehicle (saline solution) or metyrapone (Met, a common blocker of the Cort synthesis at a doses of 50mg/Kg). 24 h later, I evaluated the social fear discrimination between the groups.

Social fear acquisition

No differences in the CS-US pairings were found between the SFC$^+$/Veh and SFC$^+$/Met groups, neither with HAB rats (Fig.20 A) nor with LAB rats (Fig.20 E) (Table 20). Moreover, comparisons between breeding lines within the same treatment (*e.g.*, HAB/Met vs LAB/Met) also did not differ in the CS-US pairings (data no shown).

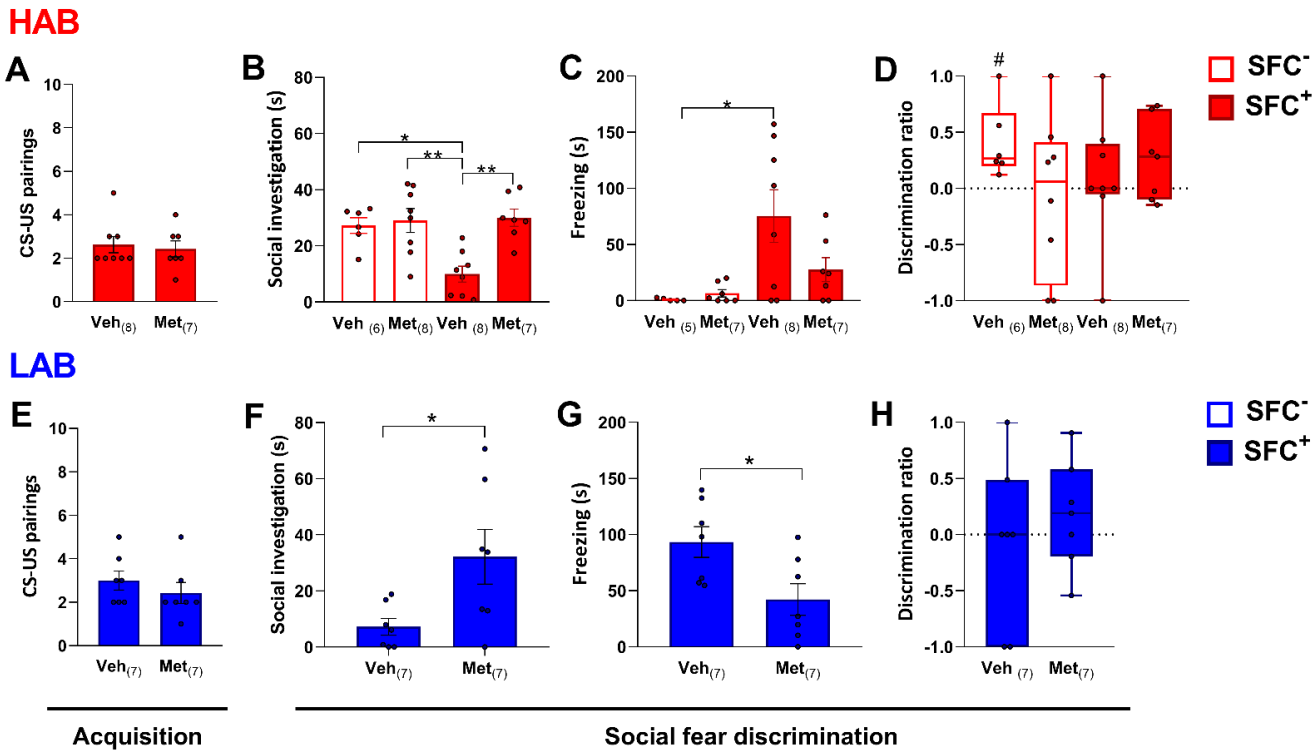


Fig.20. Social fear discrimination trial response in high (HAB) and low (LAB) anxiety-related behavior rats. A and D) Social investigation (mean ±SEM). B and E) Freezing time. C and F) Discrimination ratio (positive values indicated preference for the New stimulus). *p<0.05 and **p=<0.01 indicated comparisons between groups.

Social fear discrimination

In HAB rats, I found a significant effect of the SFC, drug treatment and interaction (Table 20). As expected, conditioned HAB rats showed a decreased social investigation (Fig.20 B) and higher freezing levels (Fig.20 C) compared to the SFC^- group (Table 20). Notably, the conditioned group treated with metyrapone did not show reduced social investigation (Fig. 20 B, Table 20). Although, the freezing levels in this group were higher (Fig.20 C). Surprisingly, only the SFC^-/Veh showed social discrimination of the Known stimulus (Fig.20 D), and the SFC^+/Veh failed to reach significant social discrimination (Table 20). Similar in LAB rats metyrapone treatment seemed to impair social fear memory consolidation, since the SFC^+/Met showed higher social investigation (Fig.20 F) and less freezing time than SFC^+/Veh (Fig.20 G). Due to the limited number of LAB animals, I could not include the SFC^- controls to confirm that indeed metyrapone restores social investigation to levels of SFC^- rats. None of the LAB groups showed social discrimination (Fig. 20 H).

Table 20. Statistical analysis of the response to SFC of HAB and LAB rats pre-treated with Vehicle (Veh) or Metyrapone (Met) (Fig. 20).

Variable	**Factor (levels)**	**Statistical**	***p* value**
CS-US pairings	HAB/Veh vs HAB/Met	U=26.50	0.895
Mann-Whitney test	LAB/Veh vs LAB/Met	U=16.50	0.347
HAB rats			
Social investigation	SFC (SFC^- vs SFC^+)	$F_{(1, 25)}$ = 5.64	0.025*
Two-way ANOVA	Treatment (Veh vs Met)	$F_{(1, 25)}$ = 10.30	0.0034**
	SFC x Treatment	$F_{(1, 25)}$ = 7.16	0.013*
Freezing time	HAB groups	H=7.27	0.064^t
Kruskal-Wallis test			
Post hoc p values		Social investigation	Freezing
Bonferroni and Dunn test	SFC^-/Veh vs. SFC^+/Veh	0.012*	0.047*
	SFC^-/Met vs. SFC^+/Met	>0.999	
	SFC^+/Veh vs. SFC^+/Met	0.002**	
	SFC^-/Veh vs. SFC^-/Met	>0.999	
	SFC^-/Veh vs. SFC^+/Met	>0.999	
	SFC^+/Veh vs. SFC^-/Met	0.002**	
Discrimination ratio	SFC^-/Veh: p=0.028		
One sample t test			
LAB rats			
Social investigation	SFC^+/Veh vs SFC^+/Met	t_7=2.44	0.044*
T test with Welch's correction			
Freezing time	SFC^+/Veh vs SFC^+/Met	t_{12}=2.61	0.022*
Mann-Whitney test			

3.4.3 Systemic and central Cort effects on the consolidation of long-term social fear memory of NAB rats

To evaluate the effects of Cort in the consolidation of social fear memory in NAB rats, I administrated Cort either systemically (ip, 5mg/Kg) or centrally (icv, 10 µg / 3 µl), including a vehicle group in each case (*i.e.,* animals received saline or ringer solution in a similar volume than Cort, respectively). After 24 h, I evaluated the social fear discrimination in the different groups.

Systemic effects of Cort

Systemic Cort application was evaluated in two cohorts with different stress conditions. In the first cohort, the rat´s handling was short (three days) and consisted of physically restrain the animal to habituated it to the injection posture (*i.e.,* each rat was hold in a supine position mimicking an ip injection). In contrast, in the second cohort, I provided a longer (5 days) and more sensible handling (*i.e*., free position and only poking their belly with the tip of a pencil mimicking the *ip* injection). When comparing the unconditioned groups from the two cohorts, I did not find any significant differences, so I pooled them in SFC^-/Veh and SFC^-/Cort. In contrast, the conditioned rats deeply differ between each other, so they were kept separately. SFC^+ rats belong to the first cohort were labelled as stressed (S): Veh-S or Cort-S, whereas those from the second cohort "less stressed" (L) as: Veh-L and Cort-L. In addition, in the second cohort I included an SFC^+ group without injection as control (NI).

During the social fear acquisition, the number of CS-US pairings was similar in all SFC^+ groups (H=2.85, p=0.583, data no shown). After 24 h, only the stressed cohort showed social fear, indicated by a reduction of the social investigation (Fig.21 A) and freezing levels (Fig.21 B) (Table 21). Moreover, none of the groups showed social discrimination (Fig.21 C, statistics no shown).

Central effects of Cort

Considering the problematic to adjust the handling and the stress due to ip injection, I decided to administrate the treatments by icv, which is painless for the animal. During the social fear acquisition, the number of CS-US pairings was similar in both SFC^+ groups (t_8= 1.313; p= 0.226, data no shown). After 24 h, both SFC^+ groups showed social fear, indicated by a reduction of the social investigation (Fig.21 D), Treatment differences were observed only in the freezing levels, where the SFC^+/Cort group showed higher freezing levels compared to the SFC^+/Veh, while the SFC^-/Veh did not showed freezing (Fig.21 E) (Table 21). Only the SFC^- group showed social discrimination (Fig.21 F, p=0.038).

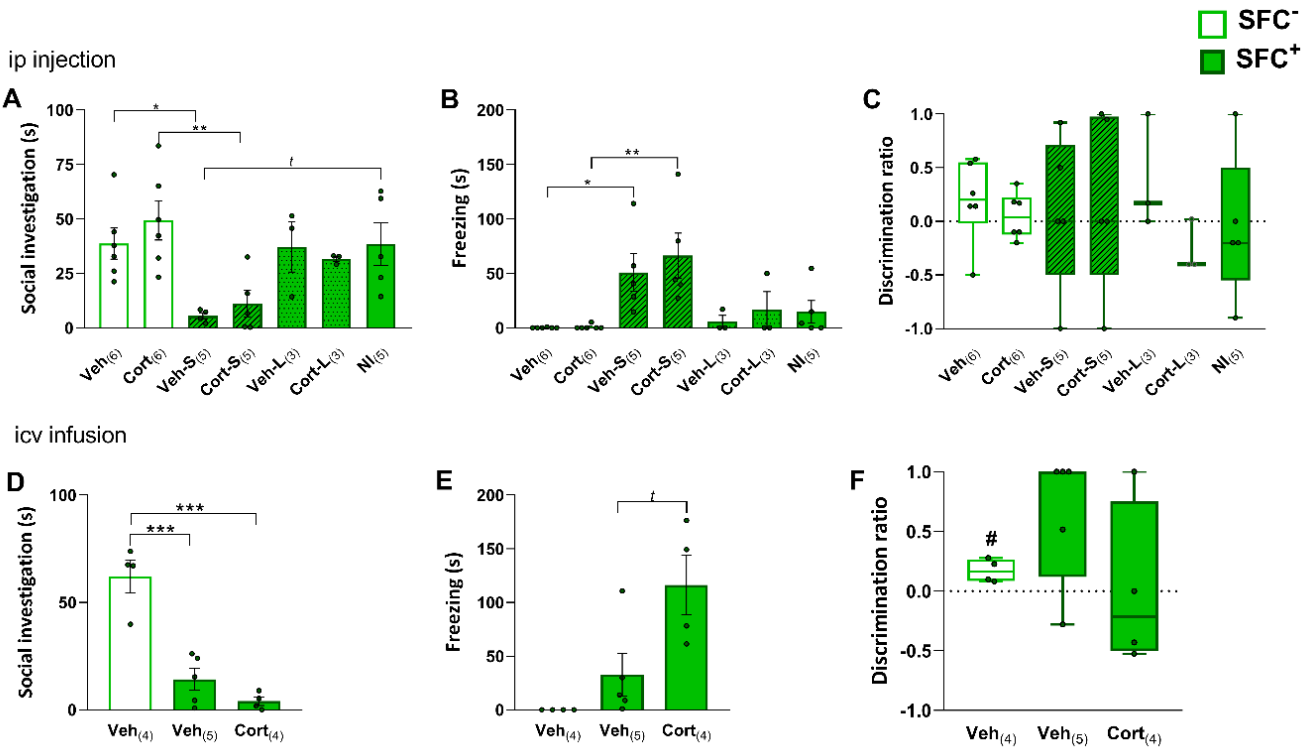


Fig. 21. Social fear discrimination trial in response to systemic (ip: intraperitoneal injections) and central (icv: intracerebroventricular infusions) Cort administration during the consolidation phase in NAB rats. The ip groups were additionally divided in stressed (bars with vertical lines, Veh-S and Cort-S) and less stressed (Dotted bars, Veh-L and Cort-L), one group did not receive any injection as control for it (NI group). A) and D) Social investigation. B) and E) Freezing time. C) and F) Discrimination ratio (positive values indicated preference for the New stimulus). *p<0.05 and **p=<0.01 indicated comparisons between groups.

Tabla 21. Analysis of the systemic and central of Cort on social fear discrimination trial in NAB rats (Fig.21)

Effects	Behavior	Statistical	*p* value
Systemic	Social investigation	$F_{(6, 25)} = 4.12$	0.005**
		Post hoc: Bonferroni's test	
		$SFC^-/Veh_{(6)}$ vs $SFC^+/Veh\text{-}S_{(5)}$	0.043*
		$SFC^-/Cort_{(6)}$ vs $SFC^+/Cort\text{-}S_{(5)}$	0.008**
		$SFC^+/Veh\text{-}S_{(5)}$ vs $SFC^+/NI_{(5)}$	0.059^t
	Freezing (s)	$F_{(6, 26)} = 4.87$	0.002**
		Post hoc: Sidak's test	
		$SFC^-/Veh_{(6)}$ vs $SFC^+/Veh\text{-}S_{(5)}$	0.020*
		$SFC^-/Cort_{(6)}$ vs $SFC^+/Cort\text{-}S_{(5)}$	0.002**
Central	Social investigation	$F_{(2, 10)} = 31.46$	<0.0001****
		$Veh_{(4)}$ vs $Veh_{(5)}$	0.0002
		$Veh_{(4)}$ vs $Cort_{(4)}$	<0.0001****
		$Veh_{(5)}$ vs $Cort_{(4)}$	0.398
	Freezing (s)	H=9.88	0.001***
		$Veh_{(5)}$ vs $Cort_{(4)}$	0.063

3.5 Brain activation during social fear discrimination in HAB and NAB rats in the Hippocampus and Amygdala

In the previous sections I provided evidence that male HAB and NAB rats are processing the social fear acquisition in a different manner, *i.e.*, NAB rats required more CS-US pairings than HAB rats to induce social fear. Furthermore, HAB and NAB rats showed a differential social fear memory consolidation. Thus, a robust long-term social fear memory was found in HAB rats, whereas a highly variable response was seen in NAB rats. Importantly, HAB rats can discriminate the known stimulus after 24 hours. To reveal possible brain regions engaged in social fear memory in HAB and NAB rats I used c-Fos expression analysis.

Therefore, I investigated if subregions of the hippocampus or amygdala were differentially activated by comparing the number of cFos-positive cells in SFC^+ and SFC^- of HAB and NAB rats. Briefly, the research design included testing the anxiety-like phenotype on the EPM, social behavior in the SPM, followed by SFC. 90 min after social fear discrimination, animals were transcardialy perfused for subsequent immunohistochemical analysis.

3.5.1 Behavioral response of HAB and NAB rats prior to the quantification of cFos levels

EPM: As expected, HAB rats spent significantly less time in the open arm (Fig.22 A) and the number of entries to all arms was significantly reduced in HAB (Fig.22 C) compared to NAB rats (Table 22). Moreover, NAB and HAB rats also differed in the latency to the first entry into the open arm (Table 22), with increased latency in HAB rats (Fig.22 B).

SPM: NAB rats showed significantly higher levels of social investigation (in s) than HAB rats (Fig. 22 D; Table 22). However, both groups showed social preference (Fig.22 E; Table 22) depicted by positive preference ratios in both lines, suggesting comparable sociability in both strains.

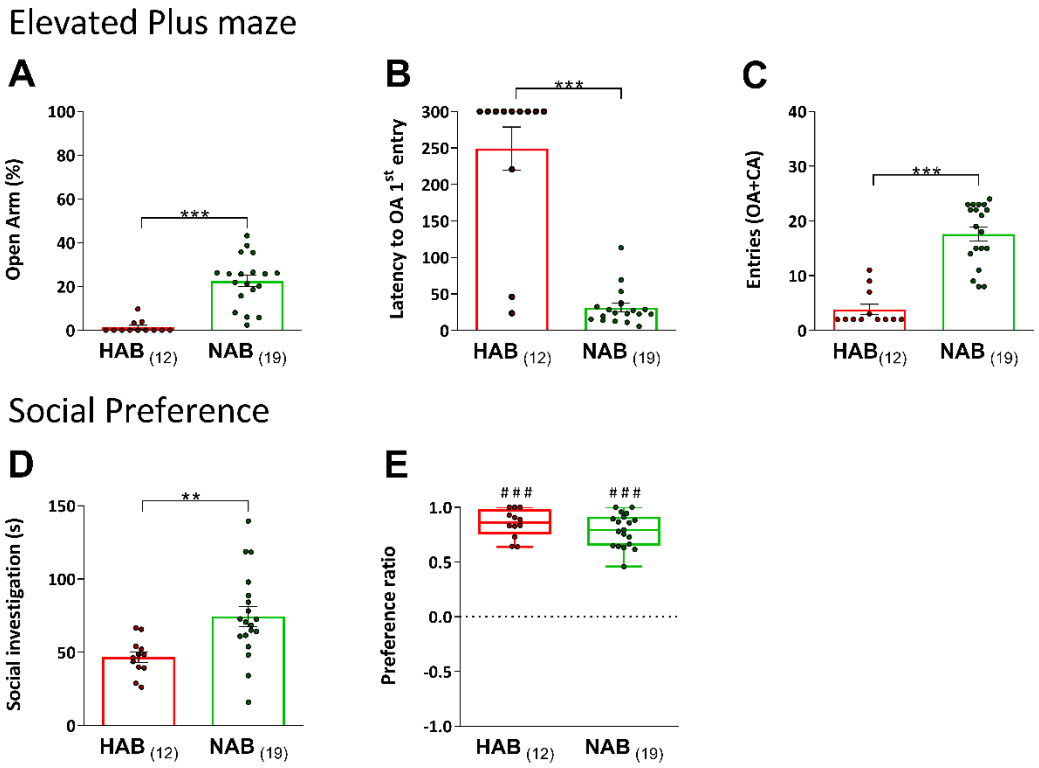


Fig.22. Anxiety-related behavior and social preference in male rats selected for high (HAB) anxiety-related behavior and non-selected (NAB) rats. EPM: A) Percentage of time spent in the open arm (OA), B) latency to enter the OA for the first time and C) total entries. SPM: D) Social investigation time (s) and E) preference ratio (calculated as: Social-Non-social time /total stimuli time). Between-group comparisons were indicated as **p<0.01 and ***p<0.001. One-sample T-test (control value zero) within each group is indicated with ###p<0.001. All data represent the mean ± SEM, except the preference ratio, which is represented the median. N-numbers for each group are given in parenthesis.

Table 22. Statistical analysis of the elevated plus maze behaviors and social preference test in HAB and NAB rats (Fig.22).

Test	Variable	Factor (levels)	Statistical	*p*-value
Elevated plus maze	OA time (%)	Line (HAB vs NAB)	U=6	<0.0001***
	Latency to OA 1st entry (s)		U=6	<0.0001***
	Entries (U Mann Whitney test)		U=13	<0.0001***
Social Preference	Social investigation (s) (Unpaired t test with Welch´s correction)	Line (HAB vs NAB)	$t_{26,14}$=3.615	0.0013**
	Preference ratio (One sample t test)	HAB	t_{11}=23.06	<0.0001###
		NAB	t_{18}=22.82	<0.0001###

Social fear acquisition

Both lines showed similar CS-US pairings (Table 23), although it was again observed that NAB rats showed higher variability regarding the number of foot shocks (mean 3.9 ±2.2, range 2 to 9) than HAB rats (mean 3 ±1, range 1 to 5). Social fear discrimination in HAB and NAB rats was measured

for each group at different time points after social fear acquisition (24 h for HAB, 24 and 6 h for NAB, respectively). Therefore, cFos analysis was performed separately for each line.

Conditioned NAB rats were fearful towards the social stimuli after 6 and 24 hours

As indication for social fear, SFC$^+$/ NAB rats showed a reduction in the social investigation compared to the SFC$^-$/NAB group (Table 23; Fig.23 A) as well as an increased freezing response (Fig.23 B, Table 23) after a 6-hours interval. The SFC$^+$/NAB measured after 24 hours, only showed reduced social investigation compared to the respective SFC$^-$/NAB group (Fig.23 A, Table 23). Further, only the SFC$^-$/NAB rats showed social discrimination (Fig.23 C, Table 23).

SFC$^+$/ HAB rats showed social fear and social discrimination after 24 hours

Similarly, to my previous results, SFC$^+$/HAB rats, showed a reduced social investigation compared to the SFC$^-$/HAB rats 24 h after social fear acquisition (Fig.23 A, Table 23), which was accompanied by social discrimination between the Know and New stimuli (Fig.23 C, Table 23). Although, SFC$^+$/HAB rats did not show significant difference in the freezing response after 24 h (Fig.23 B, Table 23), a biological trend of higher freezing time in SFC$^+$/HAB rats can be appreciated.

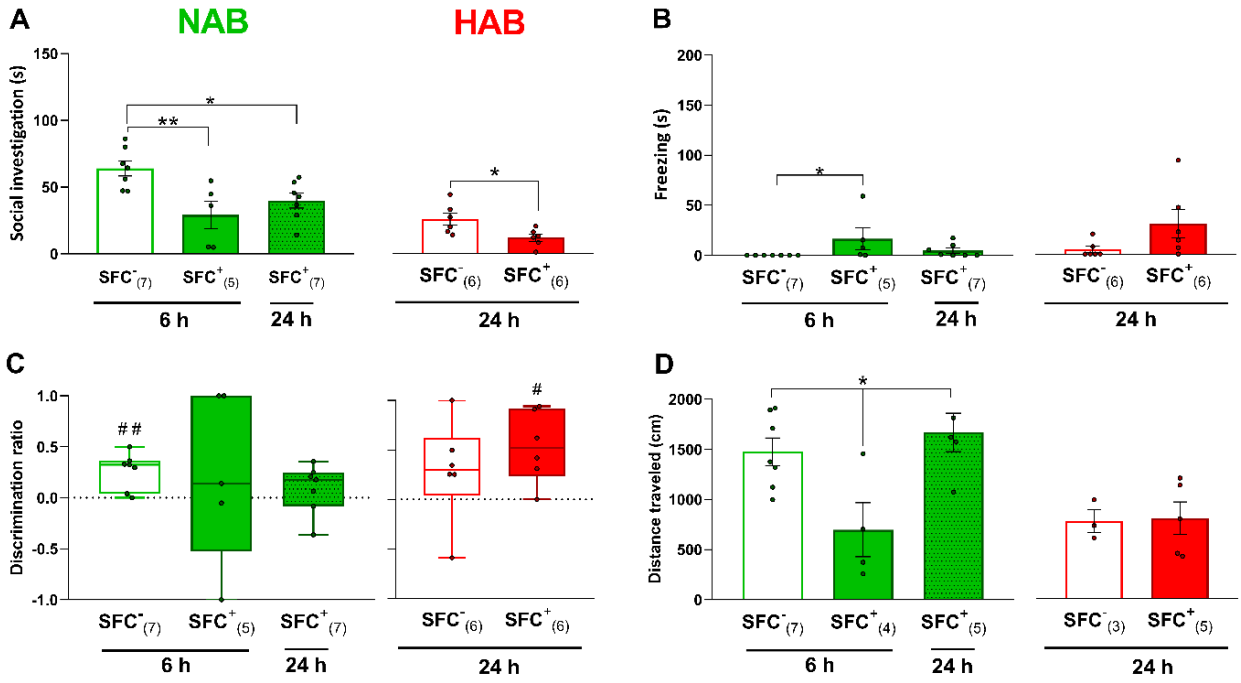


Fig. 23. Social fear, freezing, social discrimination and locomotion of high (HAB) anxiety-related behavior (24 hours after acquisition) and non-selected (NAB) rats 6 and 24 hours after social ear acquisition. A) Social investigation time (s) (mean ±SEM), B) freezing time (s) (mean ±SEM), C) discrimination ratio and D) distance travelled (mean ±SEM) of unconditioned (SFC$^-$) and conditioned (SFC$^+$) male HAB and NAB rats during social fear discrimination. *p<0.05 and **p=<0.01 indicated comparisons between groups. $^{\#}$p<0.05 and $^{\#\#}$p=<0.01

indicates significant difference with a critical value of zero in the One sample t-test. N-numbers for each group are given in parenthesis.

Table 23. Statistical comparisons for the behavioral response during social fear conditioning paradigm in in high (HAB) anxiety-related behavior and non-selected (NAB) rats (Fig 23).

Test	Variable	Factor (levels)	Statistical	*p* value	*Post hoc*	*p* value
SFC- acquisition	CS-US pairings (U Mann Whitney test)	Line (HAB vs NAB)	U=29	0.539		
Social fear discrimination	Social investigation (s) (One-way ANOVA)	HAB	t_{10}=2.59	0.027*		
		NAB	$F_{(2, 16)}$ = 6.57	0.008**	SFC^-/6h vs. SFC^+/6h SFC^-/6h vs. SFC^+/24h	0.009** 0.048*
	Freezing (s) (U Mann Whitney test and Kruskal-Wallis)	HAB	U=7	0.080		
		NAB	H=7.66	0.014*	SFC^-/6h vs. SFC^+/6h	0.017*
	Discrimination ratio (One sample t test)	HAB-SFC^-	t_5=1.39	0.222		
		HAB-SFC^+	t_5=3.59	0.015#		
		NAB-SFC^-	t_6=3.89	0.008##		
		NAB-SFC^+/6h	t_4=0.58	0.590		
		NAB-SFC^+/24h	t_6=0.95	0.376		
	Distance travelled (cm) (One sample t test)	HAB	t_6=0.12	0.907		
		NAB	$F_{(2, 13)}$ = 6.24	0.012*	SFC^-/6h vs. SFC^+/6h SFC^+/6h vs. SFC^+/24h	0.032* 0.013*

3.5.2 Brain activation in subregions of the hippocampus and amygdala after social fear discrimination

Hippocampus subregions

In the CA1 and DG subregions of the hippocampus (Fig.24 A and C), no significant differences in the number of *cFos* positive cells were observed between SFC^- vs SFC^+ groups, neither in NAB or HAB rats after 24 h (Table 24). Only the CA2/3 showed a trend indicating difference in the number of cFos positive cells between the NAB groups (Fig.24 B; Table 24). Analysis including only the groups measured after 6 h reveal a significantly decreased activation in SFC^+ group vs SFC^- group (t_{10}=2.29, p=0.044). Finally, no difference was found in CA2/3 of HAB groups (Fig.24 B, Table 24).

Amygdala subregions

No significant differences in the number of cFos positive cells were observed independent of the conditioning status (SFC^- and SFC^+) and breeding line (NAB and HAB) for any subregions of the amygdala (Fig.24 D-F, Table 24).

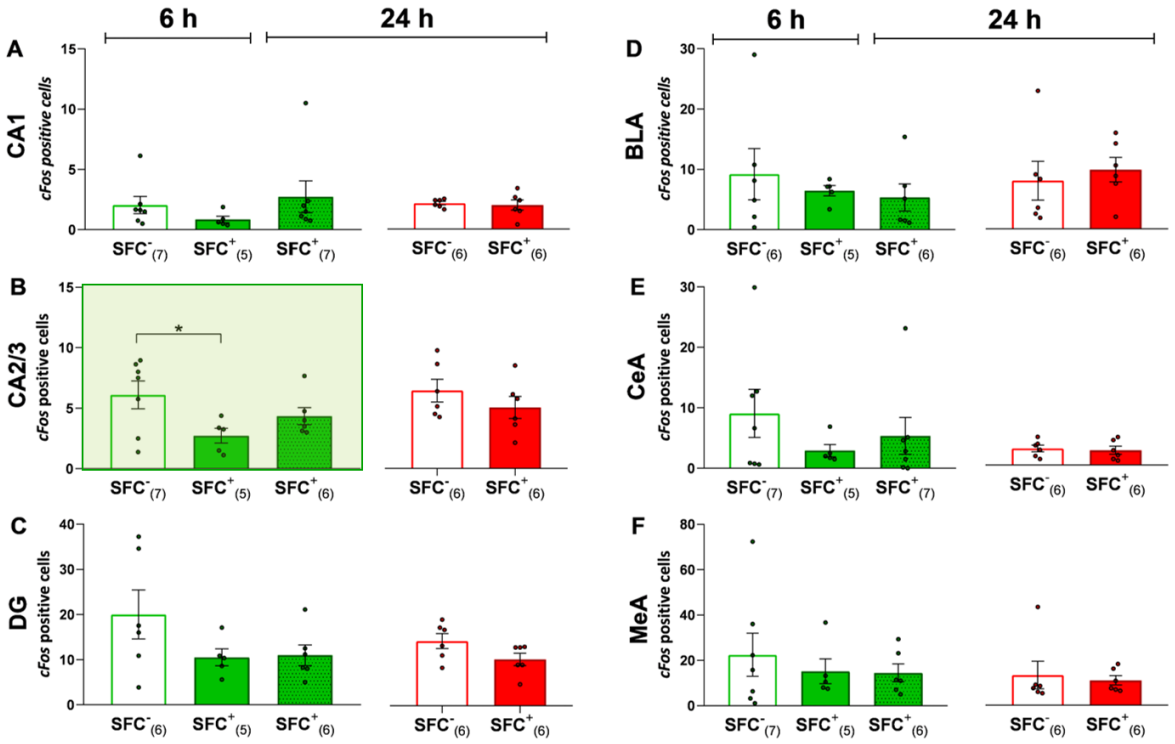


Fig. 24. Number of cFos positive cells in subregions of the hippocampus and amygdala of high (HAB) anxiety-related behavior and non-selected (NAB) rats exposed to social discrimination 6 h or 24 h after social fear acquisition. Counts of cFos positive cells in A) the *Cornu ammonis* region 1 (CA1), B) Cornu ammonis regions 2 and 3 (CA2/3), C) the dentate gyrus (DG), D) basolateral amygdala (BLA), E) central amygdala (CeA) and F) medial amygdala (MeA) of NAB and HAB rats exposed to social discrimination 6 h or 24 h after acquisition of social fear. *p<0.05 indicates differences between groups in NAB rats. All data showed the mean ± SEM and sample size for each group are given in parenthesis.

Table 24. Statistical comparisons for each brain subregions between SFC⁻ and SFC⁺ groups in high (HAB) anxiety-related behavior and non-selected (NAB) rats. HAB groups were compared by Student t-test or t-test with Welch ´s correction. NAB rats groups were compared by a One-way ANOVA or a Kruskal-Wallis test.

Brain area	Subregion	Line	Statistical	*p* value
Hippocampus	CA1	HAB	$t_{6,07}$=0.32	0.757
		NAB	H=3.87	0.146
	CA2/3	HAB	t_{10}=1.07	0.309
		NAB	$F_{(2, 15)}$ = 3.23	0.068[t]
	DG	HAB	t_{10}=1.89	0.088
		NAB	$F_{(2, 14)}$ = 2.10	0.159
Amygdala	BLA	HAB	t_{10}=0.48	0.644
		NAB	H =0.83	0.681
	CeA	HAB	t_{10}=0.34	0.738
		NAB	H =0.49	0.794
	MeA	HAB	U=14.50	0.615
		NAB	H =0.02	0.991

3.5.3 Brain activation and its relation to social fear discrimination

A linear regression analysis was carried out to test if the brain activation of each subregion significantly predicted social investigation time. For this analysis I calculated five linear regression: 1) based on the overall data (all NAB and HAB rats), 2) only in HAB rats, 3) all NAB rats (NABt), 4) only NAB rats measured after 6 hours (NAB-6h; SFC^- and SFC^+) and 5) NAB/SFC^+ measured after 24 hours. Here, I only mention the subregions in which at least one of these five regression analyses showed significant differences in the slope.

The results of the regression for the CA2/3 subregion activation did not predict the social investigation neither in the overall group nor HAB rats' response (Table 25, Fig.25 A). However, the activation of this area showed a strong trend to predict the social investigation in NABt rats and reach significance in the NAB-6h groups (Table 25. Fig.25 D). Indeed, the model in the latter groups explained a 39% of the variance in the social investigation (Table 25). The DG activation only showed a trend to predict the social investigation time base on all data together (Table 25. Fig.25 B), explaining a 12% of the variance. Finally, the only the CEA base on the overall data, explaining a 22% of the variance in the social investigation time (Table 25, Fig.25 C).

Table 25. Linear regression analyses for HAB and NAB rats

	Group	r^2	Statistical	*p* value
CA2/3	**All** (30)	0.03	$F_{(1,28)}$=0.94	0.3416
	HAB (12)	0.06	$F_{(1,10)}$=0.62	0.4504
	NABt (18)	**0.20**	$F_{(1,16)}$=4.12	**0.059**^{t}
	NAB-6h (12)	**0.39**	$F_{(1,10)}$=6.53	**0.029***
	NAB 24h (6)	0.54	$F_{(1,4)}$=4.68	0.097
DG	**All** (29)	**0.12**	$F_{(1,27)=}$3.75	0.064
	HAB (12)	0.07	$F_{(1,10)}$=0.79	0.393
	NABt (17)	0.13	$F_{(1,15)}$=2.21	0.158
	NAB-6h (11)	0.19	$F_{(1,9)}$=2.09	0.182
	NAB 24h (6)	0.20	$F_{(1,4)}$=1.02	0.370
CEA	**All** (31)	**0.22**	$F_{(1,29)}$=8.26	**0.008***
	HAB (12)	0.06	$F_{(1,10)}$=0.63	0.446
	NABt (19)	0.18	$F_{(1,17)}$=3.84	0.067^t
	NAB-6h (12)	0.18	$F_{(1,10)}$=2.14	0.174
	NAB 24h (7)	0.23	$F_{(1,5)}$=1.49	0.276

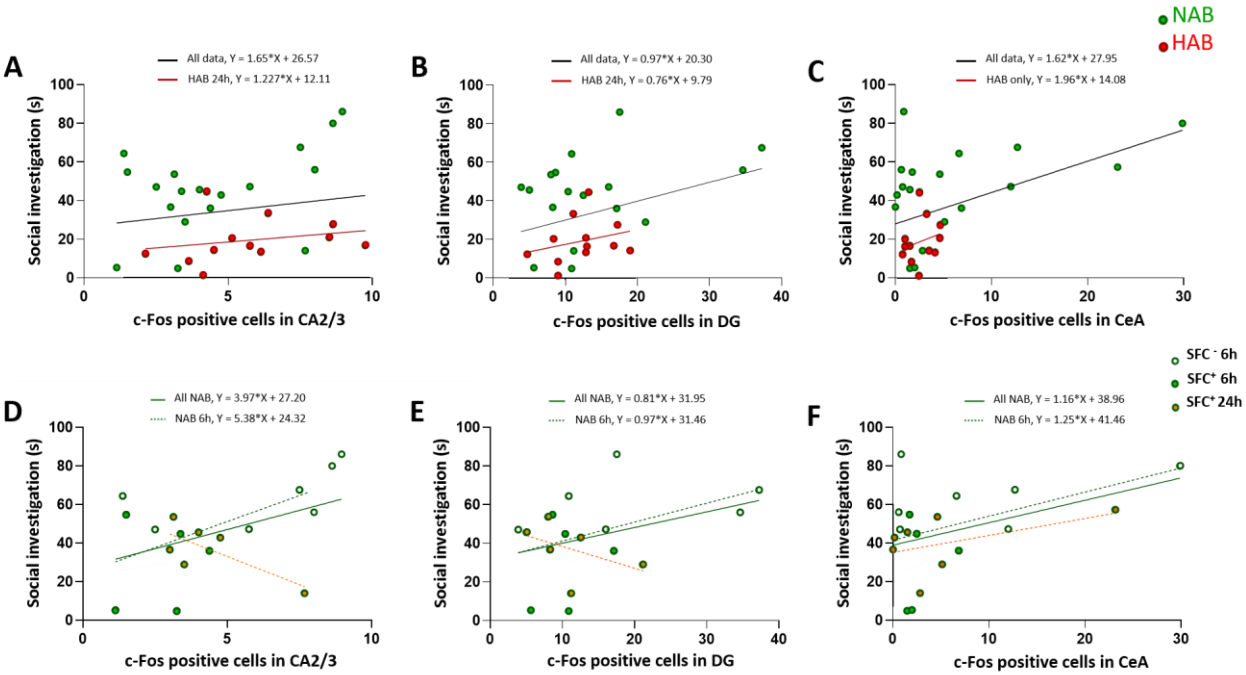


Fig. 25. Linear regression analysis by subgroups according the retrieval interval and breeding line.

Part II: Contribution of NPS to cued fear extinction and stress response in virgin and lactating females

In rats, the main source of the NPS is the LC, a key region in the stress response in which the CRF release activate the NPS neurons, and thereby triggering NPS release to downstream areas during stress responsiveness (Jüngling et al., 2012). In this regard, the NPSR are localized in strategic stress-related regions, such as the hypothalamic areas and AMY (Clark et al., 2011; Xu et al., 2007). Several stressors, e.g., acute immobilization, forced swim stress, or sleep deprivation, are known to activate the central NPS system (Adori et al., 2016; Ebner et al., 2011; Liu et al., 2011). However, a descriptive comparison of the female brain NPS system is limited so far to few studies (Germer et al., 2019; Kreutzmann et al., 2020; Wegener et al., 2012), and none of these studies considered the putative effects of the reproductive state (i.e., lactation period or estrus cycle) in the females response. Therefore, in the following experiments, I aimed to described the basal expression of the NPS/NPSR mRNA in stress-related brain regions, the effects of NPS or its antagonist in the fear extinction, and the physiological stress response (i.e., Cort and OXT plasma levels) of lactating vs virgin female rats.

3.6 *NPS* and *NPSR* expression in stress-relevant brain regions of lactating and virgin females

To investigate whether the NPS system is differentially regulated in virgin and lactating females (postnatal days 3-5), I quantified *NPS* and *NPSR* mRNA expression under basal conditions. Hence, I selected three brain areas that are part of the stress-related response: the LC, PVN, and AMY. In all selected brain regions, lactating females showed significantly higher *NPS* mRNA levels compared to virgin females (Table 26, Fig. 26 A-C). However, there were no significant differences of *NPSR* mRNA levels in any of the analyzed brain regions (Table 26, Fig.26 D-F).

Table 26. Comparison of *NPS and NPSR mRNA* levels between virgin *vs* lactating female rats.
(Unpaired t-test with Welch´s correction; *df* = degrees of freedom).

Brain region	*NPS*			*NPSR*		
	t	*df*	*p value*	*t*	*df*	*p* value
LC	2.65	15.94	0.017*	1.64	29	0.112
PVN	2.89	19.65	0.009**	0.82	23	0.419
AMY	3.26	19	0.004**	1.78	18.31	0.091

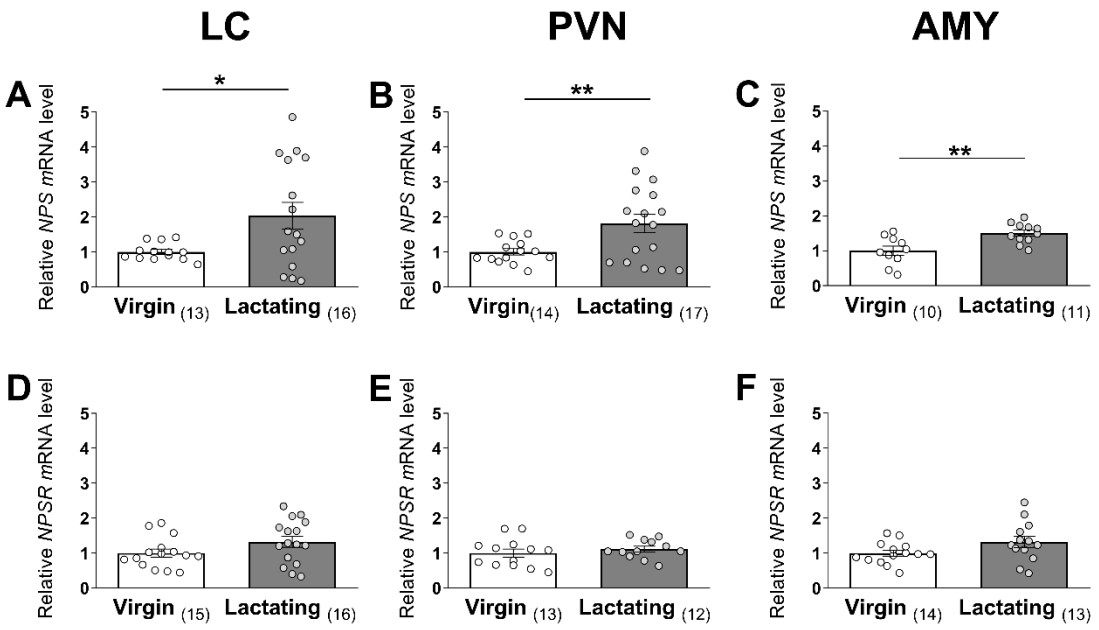


Fig. 26. Relative *NPS* and *NPSR* mRNA levels of lactating (Postnatal day 3-5) compared to virgin females under basal conditions in stress-related brain regions. Brain regions: Locus coeruleus (LC), paraventricular nucleus (PVN) and amygdala (AMY). A-C) *NPS* mRNA levels per region, in D-F) *NPSR* mRNA levels per region. Data represent mean ± SEM, in parenthesis sample size. * p< 0.05, **p<0.01.

3.7 Effects of NPS on cued fear extinction in virgin and lactating female rats

During experiment (Fig. 27 A), lactating females were isolated on the 18th day of pregnancy, as well as virgin females. Females were cannulated on the postnatal day 1 (PND 1) and after 3-4 days of recovery they were tested in the CFC. All females underwent acquisition of cued fear (PND 4-5). After 24 hours, rats were treated (icv) with either vehicle (Veh; 5 µl Ringer solution), NPS (1nmol/5µl), or its antagonist (NPSR-A, 10nmol/5 µl), and went through cued fear extinction training (PND 5-6) 20 min *post*-infusion. The next day, rats performed a cued fear recall session (PND 6-7). Additionally, I determined the estrous phase by vaginal smears of each female every day. Compelling evidence highlight the estradiol levels as a factor that affects the emotional response in females. Hence, the virgin females were grouped by **high** (**HE**, including proestrus and estrus females) and **low** (**LE**, including metestrus and diestrus) estradiol levels for the statistical analysis.

During cued fear acquisition, all females were successfully conditioned since the level of freezing increased across the time (Fig.27 B, Table 27). Surprisingly, non-significant effects of reproductive state (*i.e.*, lactating *vs* virgin females) or interaction between factors were found (Table 27). In contrast, during the fear extinction I found a significant effect of time, treatment, and a strong trend of the reproductive state in the freezing response of the females (Table 27). Moreover, no significant interactions between those factors were observed (Table 27). Regarding the time effects,

main effects post hoc analysis revealed that freezing percentage decreased after the third CS presentation (Table 28). Within groups, lactating, and LE virgin groups (Fig. 27 C and D) showed the same trend, however, in HE virgin females the freezing percentage seems to be remained uniform (Fig. 27 E).

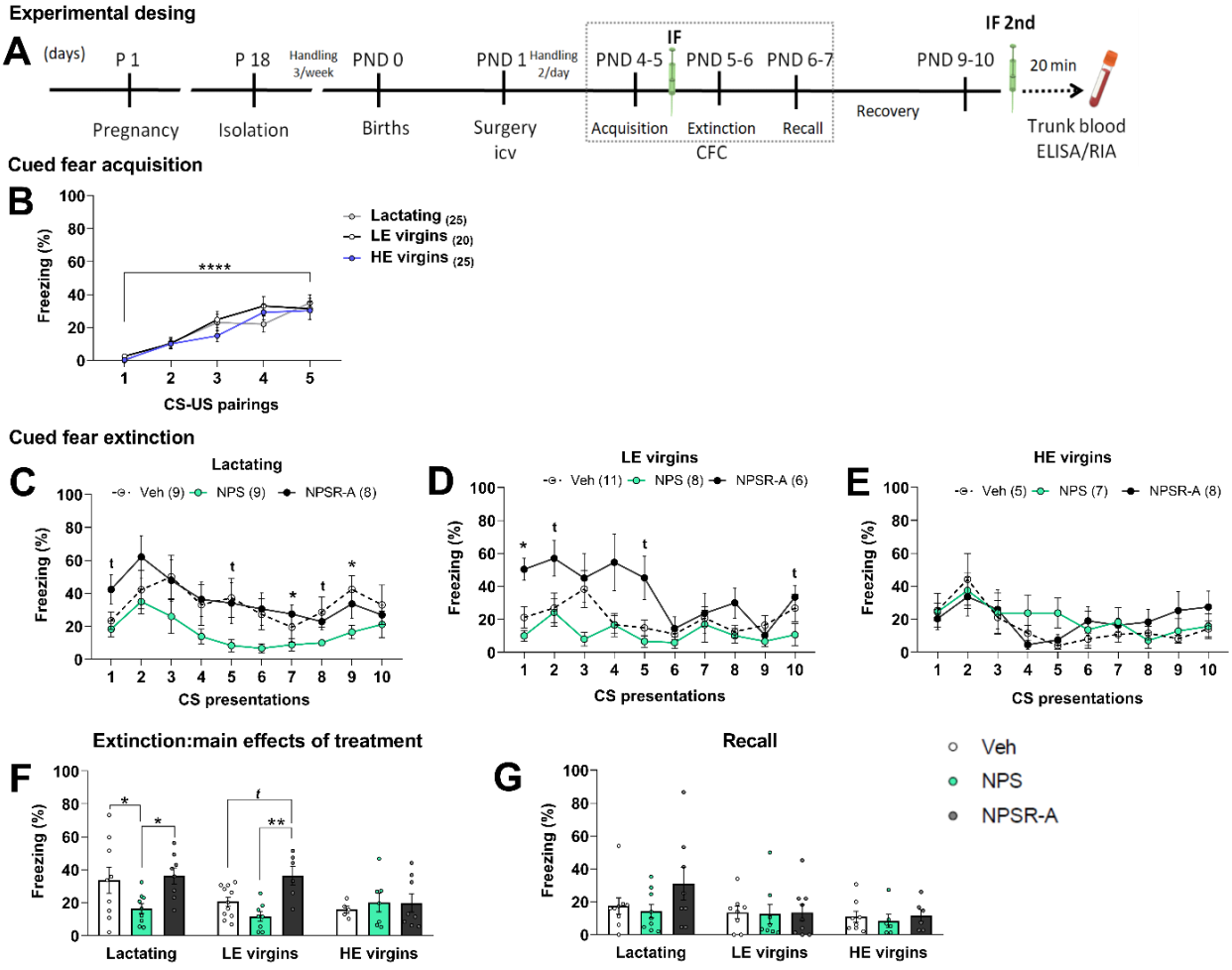


Fig.27. Percentage of freezing response of Lactating *vs* virgin (high or low estradiol levels) rats to the cued fear conditioning paradigm (CFC). A) Experimental design: virgin females were split in high (HE, females in estrus and proestrus) and low estradiol levels (LE; females in metestrus and diestrus). PND: postnatal day, IF: *icv* infusion of either vehicle (Veh, 5µl Ringer solution), NPS (1nmol/5µl) or NPS receptor antagonist (NPSR-A, 10nmol/5µl). B) Cued fear acquisition. Cued fear extinction per group in: C) lactating, D) LE virgin, and E) HE virgin females. F) Main effects of treatment during cued fear extinction. G) Recall of cued fear. All data represent the mean ±SEM. In parenthesis sample size per in group. Group comparisons: *p<0.05, ** p<0.01; ^{t}p<0.07.

Post hoc analysis for the main effects of the treatment (Fig.27 F) showed that NPS treatment in lactating females leads to a significant reduction of the freezing response compared to vehicle and NPSR-A-treated lactating females (Table 29). The LE virgins treated with NPSR-A showed higher

levels of freezing compared to respective vehicle and NPS treated virgins (Table 29). In contrast, the HE virgin females did not show a significant response to the different treatments. These patterns were the same across the CS presentations, Lactating/NPS females shoed a reduced freezing response compared to Veh rats in the CS 7 and CS 9 ($^{*}p$<0 05); and a trend in the CS 1, CS 5 and CS 8 (^{t}p <0.07) (Fig.27 C). In contrast, LE virgin/NPSR-A females showed a delayed in the extinction compared to Veh group (CS 1: $^{*}p$<0 05, CS 2, CS 5, and CS 10: ^{t}p <0.07) (Fig.27 D).

Table 27. Statistical analysis between lactating and virgin female rats in the Cued fear conditioning (Fig. 27)

Test	Factor (levels)	Statistical	*p* value
Acquisition	Time (CS-US)	$F_{(3.012, 204.8)} = 35.89$	<0.0001****
	Reproductive state	$F_{(2, 68)} = 0.46$	0.634
	Interaction	$F_{(8, 272)} = 0.97$	0.461
	Post hoc Bonferroni´s test	cs-us1 *vs* cs-us5	0.0001****
Extinction	Time (CSx10)	$F_{(4.05, 251.1)} = 9.94$	0.001***
	Reproductive state	$F_{(2, 62)} = 3.09$	0.052^{t}
	Treatment	$F_{(2, 62)} = 6.40$	0.003**
	Time x Reproductive state	$F_{(8.10, 251.1)} = 1.05$	0.396
	Time x Treatment	$F_{(8.10, 251.1)} = 0.52$	0.845
	Reproductive state x Treatment	$F_{(4, 62)} = 2.19$	0.081
	Time x Reproductive state x Treatment	$F_{(16.2, 251.1)} = 1.24$	0.235
Recall	Time (CS-US)	$F_{(2, 62)} = 1.19$	0.311
	Reproductive state	$F_{(2, 62)} = 2.94$	0.059
	Treatment	$F_{(4, 62)} = 0.74$	0.566

Table 28. Paired comparisons between CS presentations: *p* values (Bonferroni multiple test).

	CS 1	CS 2	CS 3	CS 4	CS 5	CS 6	CS 7	CS 8	CS 9	CS 10
CS 1		**<0.001**	1.000	1.000	1.000	**0.019**	1.000	0.133	1.000	1.000
CS 2			0.134	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.028**
CS 3				0.213	**0.029**	**<0.001**	0.180	**0.036**	.403	1.000
CS-4					1.000	0.261	1.000	1.000	1.000	1.000
CS-5						1.000	1.000	1.000	1.000	1.000
CS-6							1.000	1.000	1.000	1.000
CS-7								1.000	1.000	1.000
CS-8									1.000	1.000
CS-9										1.000
CS-10										

Table 29. Comparisons within groups for the main effects of treatment
(Tukey's multiple comparisons test, *p* values)

	Lactating females		High estradiol (HE) virgin females
Veh *vs* NPS	0.030*	0.353	0.865
Veh *vs* NPSR-A	0.914	0.073 [t]	0.873
NPS *vs* NPSR-A	0.013*	0.005**	0.999

During recall of cued fear (Fig.27 G), some virgin females had a shift of their estrus phase, therefore the estradiol category varied for those rats. Thus, to avoid confounding factors those females that swap categories (*i.e.*, from high to low estradiol state or vice versa) were omitted from the analysis of the freezing response during recall. No significant effects were observed in treatment or reproductive state (Table 27).

3.8 **Physiological response to central NPSR activation and blockade**

To further enhance our knowledge of the physiological response to central NPSR activation and blockade in female virgin and lactating (PND 9-10) rats, all animals were infused (icv) with the same substance for a second time, and sacrificed 20 min later. Then, I collected trunk-blood to measure peripheral levels of Cort, OXT, and NPS. Due to the reduced group size of especially virgin females, no comparison between low and high estradiol levels was performed. Statistical analysis revealed a significant effect of treatment ($F_{(2, 50)}$ = 7.93; *p*=0.001), but not reproductive state ($F_{(1, 50)}$ = 2.16; *p*=0.148). In detail, NPS infusion significantly increased plasma Cort levels in virgin females compared to Veh and NPSR-A-treated rats (Fig.28 A). In addition, peripheral OXT and NPS levels were determined in a subset of samples (N=5 per group) due to restricted availability of the OXT RIA and the ELISA for NPS analysis. These analyses reveal a significant effect of treatment ($F_{(2, 26)}$ = 18.82; p<0.001) but no reproductive state effect was observed ($F_{(1, 26)}$ = 0.06; *p*=0.809). Bonferroni's multiple comparisons test (Table 30) revealed that OXT levels raised after NPS infusion in both, lactating and virgin females when comparing to respective Veh and NPSR-A treatment (Fig.28 B). In contrast, no differences were detected in plasma NPS levels of any treatments or reproductive state (Fig.28 C).

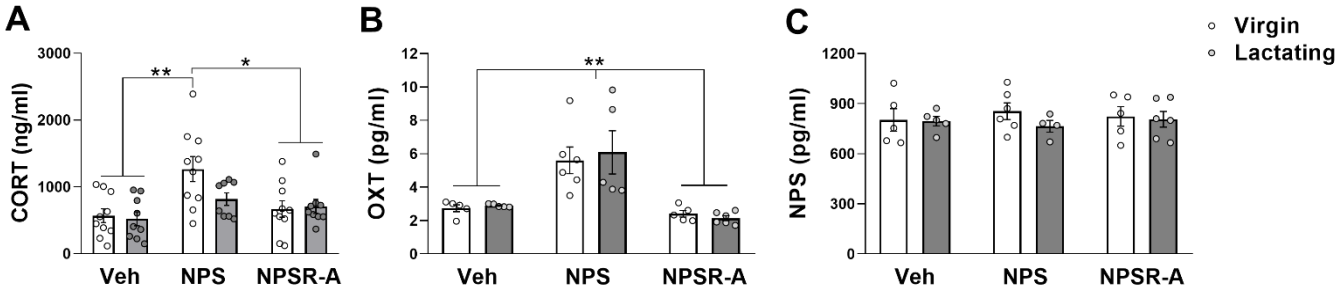


Fig.28. Physiological response of corticosterone (Cort), oxytocin (OXT) and neuropeptide S (NPS) after central activation and blockade of the NPS receptor (NPSR-A) in lactating and virgin females. Females were infused either with vehicle (Veh, 5µl Ringer solution), NPS (1nmol/5µl) or NPS receptor antagonist (NPSR-A, 10nmol/5µl). In A) Plasma Cort levels (N=10 per group), B) OXT (N=5 per group), and C) NPS levels (N=5 per group) Data represent mean ±SEM. Between group comparisons *p<0.05, ** p<0.01.

Table 30. Bonferroni's multiple comparisons test of plasma OXT levels

	Virgin	Lactating
Veh vs NPS	0.009**	0.006**
Veh vs NPSR-A	>0.999	>0.999
NPS vs NPSR-A	0.004**	0.001**

In addition, I analyzed dynamic response of plasma Cort levels to central activation in virgin females and blockade of the NPS (NPSR-A) in lactating females (lactation day 3-5). Following recovery from the implantation of an icv cannula and jugular vein catheter, I collected blood samples in different time points: two basal samples (separated by 30 min each), and three post-infusion samples after 15, 25, and 35 min. As a mild stressor, animals were placed in the elevated platform (EP, for 5 min) after the 15 min-sample. Cort level were normalized to the average of both basal samples in each group. Virgin females treated with NPS showed significant higher Cort levels compared to the vehicle group at 15 (t_9=2.91, p=0.017*) and 25-min (t_9=3.39, p= 0.008**) post-infusion samples (Fig.29 A). Moreover, only the NPS-virgin group experienced a significant increase of Cort levels after 25-min compared to their basal state (t_5=3.09, p=0.027). In contrast, lactating females did not show treatment or stress effects (Fig. 29 B, data not shown).

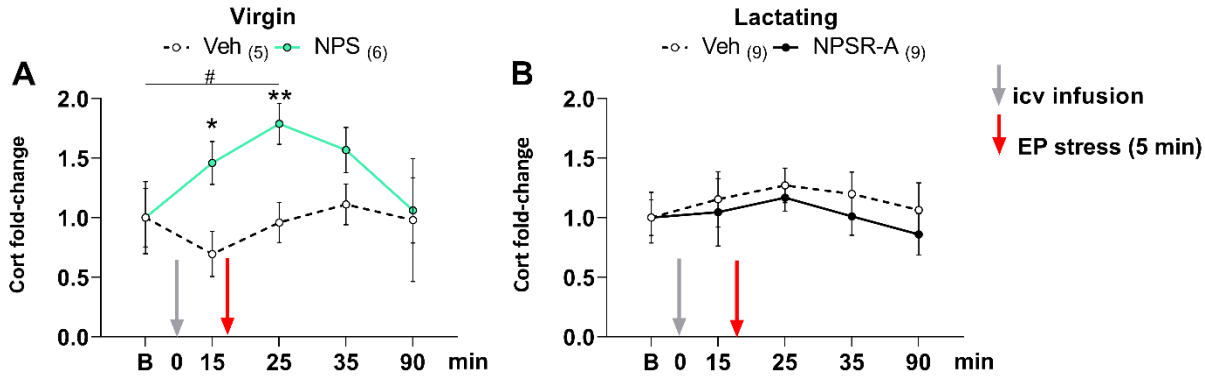


Fig.29. Dynamic corticosterone (Cort) response after central activation of the NPS in virgin females and blockade of the NPS receptor (NPSR-A) in lactating females. Cort fold-change was normalized by the basal level mean (**B**) for each time point measured. The gray arrow indicated the icv infusion in each group and the red arrow the stress in the elevated platform (**EP**, during 5 min). In A) Virgin females treated with NPS (1nmol/5µl) or vehicle solution (N=5-6 per group), B) Lactating females treated with NPS receptor antagonist (NPSR-A, 10nmol/5µl) or vehicle solution (N=9 per group). Vehicle groups (Veh) received 5 µl of Ringer solution. Between group comparisons: *p<0.05, ** p<0.01 and within group comparisons across time points: $^{\#}$p<0.05.

Discussion

4. Discussion

Part I: Social Fear Conditioning in rats

4.1 **SFC protocol adjustments: on the basis of species**

The SFC was developed as a mice model, conceived to study the neurobiological bases of social fear against conspecifics and to contribute to a better understanding of pathologies such as the SAD in humans (Masis-Calvo et al., 2018; Toth et al., 2012b, 2013). Social fear behaviors are generally similar in mice and rats (Blanchard et al., 2001a); however, some species-specific differences can be found not only at a behavioral (rats displayed more affiliative behavior than mice), but also a neurobiological level (see a detailed review in section 4.1.4). Moreover, rats offer some technical advantages due to their more complex behavioral repertoire and their larger size compared to mice, *(e.g*., surgical procedures, fMRI availability, and less sensitivity to hepatotoxicity during chronic drug administration; reviewed in (Ellenbroek and Youn, 2016). Beyond the "*pros* and *cons*" of choosing one species over the other, the putative species-specific differences might also give us relevant insights to uncover the mechanisms that regulate social fear. Therefore, the main goal of part I of my thesis was to extend and validate the SFC paradigm to male rats. In the following sections, I will examine the modifications of the SFC protocol implemented in male rats. The order of the following sections is organized according to the main topics involved in the SFC process, such as the acquisition of social fear, its discrimination, the retrieval-interval effects in NAB rats, and a finally, the discussion of the relevant species-specific differences that may account for the differential responses between mice and rats in this SFC paradigm.

4.1.1 Features of social fear acquisition in rats

Shock intensity and number of CS-US pairing differed between rats and mice

Shock intensity

I modified the shock intensity with two purposes, first to promote a robust social fear expression in rats, and second to reduce the number of shocks that each rat received. For instance, with 0.7 mA rats received up to 12 shocks to reach the conditioned criteria (*i.e*., rats remain away of the conspecific up to 5 min). In Fig. 9, I showed that neither 0.7 nor 0.8 mA promoted any sign of social fear. Finally, I set the intensity at 1mA based on the finding that independent of the number of CS-US pairings rats reached a plateau effect of fear response (*i.e*., freezing response) at this intensity (Fanselow and Ponnusamy, 2008). Nevertheless, I cannot directly dissect the contribution of the

shock intensity in this case because: i) animal cohorts were evaluated in separate occasions, and *ii)* in the meantime, I changed other testing features (those will be discussed below) that led to a more robust social fear response in rats. However, a positive relation between shock intensity and robust response in a passive avoidance conditioning was proved by Ader and colleagues using a "*step-down*" task (Ader and de Wied, 1972). They found that each increase in the shock intensity occasioned an increase in the latency to step down (*i.e.*, an indicator of better learning). Those effects were independent of the shock duration (Ader et al., 1972). As an additional observation, the shock intensity is a sensitive parameter from the perspective of animal welfare, and it should be considered to avoid unnecessary induction of suffering.

Number of shocks

Mice need on average 2±1 shocks (maximum 5 shocks)(Toth et al., 2012b), while rats reported 3±2 shocks (maximum 12 shocks), which varied according to the breeding line analyzed (Fig. 15). This difference between species may have produced the larger individual differences in the fear response and the consistency of the model in rats. In fact, the variability of the shocks' density could have affected the reliability to predict the contingency and the CR retention in rats, which also varied between the lines (Fig. 30).

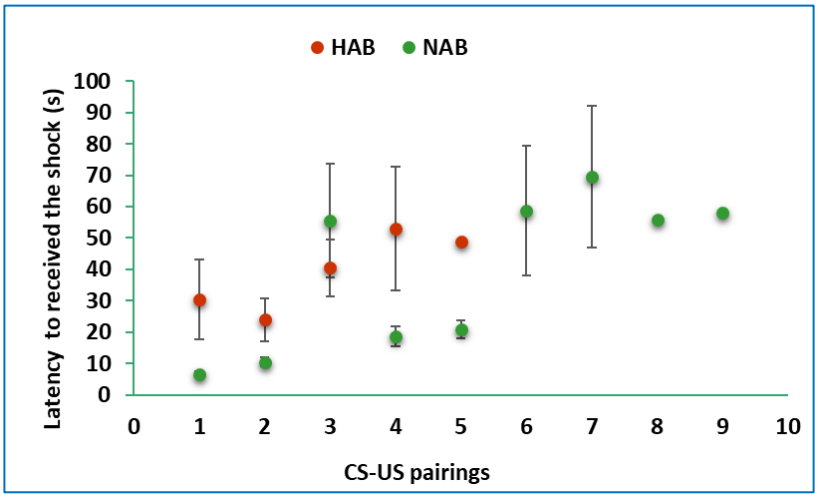


Fig.30. Inter-shock variability between HAB and NAB rats. Although it was assumed a similar acquisition based on the average number of shocks, the latency and interval variability depict a more complex fashion. Latency showed from the moment the social stimulus is presented. Based on data from the cFos experiment (Results section 3.5).

There are also constitutive differences in the defensive repertoire between rats and mice. For instance, mice showed more risk assessment to highly threatening stimuli than rats (Blanchard et al., 2001a). This may prevent mice from receiving more shocks compared to rats. In addition, the level of pro-sociality varies between these species. Rats witnessing a conspecific restrained in a cage used to display cooperative behaviors contributing to free up the conspecific (Bartal et al., 2011). This behavior occurred even when another rewarding response was also possible (*i.e.,* a rat could choose between open a cage with chocolate or release a conspecific). Interestingly, rats ended up releasing the partner and sometimes shared the chocolate with it (Bartal et al., 2011). Therefore, one putative reason behind the high number of shocks received by the rats during conditioning reflect this cooperative effort to release their conspecific. Finally, it cannot be ruled out putative differences in pain sensitivity between species (see for review (Mogil, 2019).

Specificity of CS-US

The acquisition of social fear is based on the association between conspecific exploration (CS) and an aversive electric foot shock (US). In the SFC mouse model, this association reliably leads to social fear expression at 24 h and 3 weeks after acquisition (Toth et al., 2012b). Toth and colleagues provided a straightforward experiment demonstrating that the fine-tuning between conspecific exploration and its punishment by a shock did induce social fear rather than the shock on its own. My findings not only reproduced this observation in rats but also extended it (Fig.12 A). I provided a more appropriate control for the CS-US specificity, namely, the unpaired group. The unpaired group was exposed to all the conditions that an SFC^{+} group experienced except the US-CS pairing, *i.e.,* each subject received four random shocks without punishing the conspecific exploration. Indeed, the unpaired group did not differ from the SFC^{-} group in the social investigation time (Fig. 12 B). Moreover, only the R-SFC^{+} significantly show a decreased social investigation compared with the other groups. Although the unpaired groups showed general freezing (Fig. C), this did not interfere with its social engagement. Control for the specificity aspect was especially relevant since previous studies showed that electric foot shock exposure decreased social investigation in a novel environment, *i.e*., similar to SFC protocol in rats (Haller et al., 2003; Short and Maier, 1993; Sigmundi et al., 1980). However, it must be noticed that those protocols applied up to 10 shocks in unescapable situations and at intensities varying between 0.8 and 3 mA. These extreme conditions may have resulted in a “helplessness learning" state instead of a simple fear conditioning.

Notes about pre-conditioning habituation and absence of the empty cage during consolidation period

Pre-exposure effect

The first step in the SFC in rats comprised a 1-min habituation to the conditioning chamber (one day before the acquisition). This procedure was added to facilitate the exploration of non-social and social stimuli, especially in the HAB rats. However, in the literature of fear conditioning, it has been largely known that **pre-exposure** to the conditioning stimuli (the chamber form part of the contextual memory) will reduce the magnitude and efficiency of associative learning, that is, the expression of social fear. This might be even more important for the NAB rats, which showed higher variability in the conditioned response. For instance, some NAB cohorts showed a robust memory up to 6 hours (Fig.10), whereas other cohorts did show long-term memory (Fig.24). Thus, direct comparisons are warranted to clarify the potential effect of chamber pre-exposure between the rat lines.

Moreover, this principle of "pre-exposure" can also apply to the empty cage presentation at the beginning of the conditioning, and even more relevant to the use of a rat as a CS. To extend the latter idea, let us consider the rats experience previous to the SFC: their "standard housing" (in most of the laboratories) is a group of 3-4 males, they probably spend together more than a few weeks that allowed a stable hierarchy, unless the individuals were mixing on purpose. In these conditions the interaction with a conspecific would naturally and constantly pairing the social investigation with positive consequences (*i.e.*, allogrooming or play behavior), in other words the "pre-exposure effect" to the CS. Even though the rats were isolated three days prior the SFC, this do not change the positive consequences predict by the CS. During the SFC we are trying to reverse this positive pairing with a few sessions, which sound as "hard" task, and may explain the high variability in the necessary CS-US pairing in rats (3-5 shocks in general). -At this point you may thing, I am my own devils' lawyer -. However, even difficult my results proved that against the odds it is possible, and as well explain the higher variability that accompanied the social fear discrimination responses. Finally, if you analyzed in the same way the "pre-exposure" in mice, it also may explain why the model is more robust in them. Male mice are more territorial, often fight until extreme consequences, and this may end in isolation as a housing condition practice in many labs.

Absence of the empty cage during the consolidation period

Initially, in the mice protocol, animals were exposed to an empty-cage overnight period to prevent developing fear responses to the cage. This adaptation was based on the observation that an empty cage may become conditioned during the procedure (Toth *et al.,* 2013). Nevertheless, the same procedure in rats could negatively impact the rat's social recognition abilities due to the "*retroactive interference*" phenomenon (see a review in (Camats Perna and Engelmann, 2017)). Briefly, the **retroactive interference** occurs when another stimulus is presented during the consolidation period. The presentation of different familiar stimuli confuses which pieces of information should be learned from that task (e.g., imbuing a conspecific with the aversive properties of the shock) and which stimulus should become conditioned. Thus, during the consolidation period, the specificity of the social memory could be weakened. Therefore, I decided not to expose the rats to the empty cage overnight.

4.1.2 Evaluation of social fear in rats

Context to evaluate the social fear: when, where, and with whom?

When?

During the establishment of the SFC protocol, I also tested if the light-dark period had an effect (Fig. 9). I did not find differences between the light and dark period with the old SFC setup. Notably, rats seem to show better retention of passive-avoidance learning in the light phase of the circadian cycle (Davies et al., 1973). I decided to keep similar conditions as the ones used in the mouse model, because it may facilitate future comparisons between species. Also, shifting the light-dark cycle may disturb the memory process in rats (Fekete et al., 1985).

Where?

It is well-known that environmental factors influence social behaviors (reviewed in (Litvin et al., 2008)). For instance, novelty, open spaces, bright lights, and loud noise can increase anxiety states and dampen social interaction. To assess the influence of some environmental conditions. I evaluated how different contexts affected the "social fear discrimination." In that regard, I compared the response in the home cage (the original protocol in mice) *vs* a novel arena (to stimulate exploration), but no differences were observed (Fig. 9). The final settings of the protocol took place in a big arena because a larger distance between the stimuli enabled us to see clear preferences. Familiarity with the test arena significantly reduced levels of anxiety, *i.e*., by increasing

interaction. Thus, I intentionally combined the social preferences test (SPM) in the same arena one or two days before the SFC, because that session served as a habituation trial as well (*i.e*., the SPM lasted up to 8 minutes). Finally, the SFC protocol in rats included a pre-trial (4-minutes) of habituation. These proved to be enough in the new protocol, since all SFC groups showed higher exploration levels in all experiments of part I.

To whom?

In rats, social fear was more robust against the conspecific used as a stimulus during social fear acquisition, called the Known. The latter is in line with data from acute social defeat in golden hamsters and rats, which also induced specific avoidance against the dominant (Lai et al., 2005; Lukas et al., 2011). Notably, chronic social defeat leads to a general avoidance of conspecifics, suggesting that the levels of stress and the social recognition follow the Yerkes and Dobson law. Another possibility behind that enhancement of social memory is the "relevance of the stimulus". Traditionally, social memory studies use same-sex juveniles or ovariectomized (OV) female adults as stimuli, mostly to avoid either aggression or mating episodes.

The relevance of social recognition between adults comprises a broad spectrum of social aspects, such as territoriality, social hierarchies, nepotistic contexts, and reciprocal altruism (Holmes and Mateo, 2007). Previous studies in our lab proved that male rats retained up to 2 hours the memory of a female conspecific compared to a juvenile (Lukas et al., 2013). In terms of sex differences, adult male rats prefer to explore juvenile rats while avoiding adult counterparts (Rogers-Carter et al., 2019). In contrast, female rats show little interest in infants until before parturition (Insel and Fernald, 2004). Sex differences in social recognition in rats were linked to OXT and AVP (Lukas and Neumann, 2014). One extreme example of the relevance of the stimulus was observed in lactating females, I unsuccessfully tried to conditioning mothers against their babies (N=5), I stopped the procedure after 12 shocks to preserve animal welfare (Personal observation). This evidence opens future experiments in which different social stimuli (juveniles, opposite sex, defeater) should be included as stimuli in the SFC.

4.1.3 SFC protocol prolong the social recognition in NAB rats up to 6 h

I conditioned NAB rats in the final version of the adapted SFC protocol, to evaluate the effect of three retrieval intervals, specifically at 2, 4, and 6 h after social fear acquisition. Here, I found that learning in SFC induced a social fear memory that lasted up to 6 hours in NAB rats, including reduced

social investigation and high freezing levels. However, in longer retrieval intervals in NAB rats (24 h), did not show robust social fear. Taking together these two observations, it seems that NAB rats fail to consolidate long-term memories. Recently, a SFC study in mice described that the induced social fear in this paradigm required of two protein stages, the first starting right after the acquisition, while the second stage occurred after 6 h (lasting circa 5 h) (Kornhuber and Zoicas, 2020). It is plausible that rats and mice differ in the mechanisms that take place in the second stage of protein synthesis that led to long-term social fear memory consolidation. Moreover, the second stage timepoint seem to vary depend on the memory type, for instance in social memory last between 6 to 18 h after sampling (Richter et al., 2005; Wanisch et al., 2008), and in the inhibitory avoidance memory from 3 to 7.5 h (Grecksch and Matthies, 1980; Igaz et al., 2002; Quevedo et al., 1999). The latter range of second stage of protein synthesis seem to fit better with the data obtained at 4 h, explaining why the freezing differences and the social discrimination were weaker (*i.e.*, memory is in a unstable period) compared with the other intervals. Surprisingly, the SFC^- groups showed significant social discrimination at 2, and 6 h. Social memory in rats (neutral, non-adverse) last up to 45 min in males, and up to 2 h in females (Camats Perna and Engelmann, 2017). Thus, the SFC seems to extended the non-adverse social memory, this may be explained by an enhanced effects of the GC, since the arousal in the SFC^- group is almost the same as in the SFC^+ groups (Fig.19).

4.1.4 The modified technical settings reflect the species-specific differences

The acquisition and expression of social fear proved to be species-specific in the SFC, mice showed a generalize and long-term social fear (24 h), while rats showed higher variability either in the level of social discrimination as well the retention of the social fear memory depends on the anxiety-related profile (see section 4.2 below). Here, I discussed a few more aspects that may contribute to understanding the SFC protocol changes in rats and the behavioral outcomes between these species.

Olfactory signaling differences between rats and mice

Mice social recognition can be achieved by both volatile and non-volatile components of their odor signatures, whereas rats used to require non-volatile components (Noack et al., 2010). This is a critical difference during the social fear acquisition between mice and rats, as they differed drastically in the sampling time. For rats, the access to non-volatile odors (mainly by anogenital exploration) is first restricted by the enclosure of the social stimulus, and second by the obvious

punishment effects. Although mice face the same constraints, their sampling is not limited to the direct exploration, and the 3-7 min aftershock may represent a constant exposure to the signature odors.

Sociality levels

In light of the consequences of an aggressive encounter between rats and mice, it would be easy to understand why mice's social fear response is long-lasting (*i.e*., 24 hours to 15 days) and generalized (independent of social recognition) as compared to rats. In mice, aggression and territoriality are hardwired to be constantly expressed upon changes in the environment or group members (called "demes" its typical structure consists of one male that mates with several females) (Van Zegeren, 1979). Schmid-Holmes *et al.* (2001), showed that mice burrows are less complex than rat burrows, and again male mice tend to occupied a single cavity which reduced male to male interaction (Schmid-Holmes et al., 2001). In the laboratory context, adult male mice must be single housing to prevent killings, which resemble the evolutive trait of their natural social interactions.

In rats, aggression may be initially necessary to obtain a dominance-subordinate relationship. Once it is established, a stable hierarchy suppresses further aggression and unwanted fights among group members (Blanchard et al., 2001b). Another aspect to consider is the impact of the ultrasonic vocalizations (UV) between the species. Rats display alarm (22-kHz) and prosocial (50-kHz) vocalizations, which prevent continuous aggressive behaviors. Rats are highly social animals that spend much time in affiliative interactions and emitting high rates of 50-kHz calls. Moreover, the majority of rats readily engage in social behavior and find it is rewarding, whereas mice showed less time interacting with a conspecific, and many even find it aversive (reviewed in (Ellenbroek and Youn, 2016)). Altogether, it supports the idea that mice are easier to social fear conditioning than rats.

4.2 **Innate anxiety-related behavior influences susceptibility social fear conditioning**

Conditioning events and the BI trait to unfamiliar situations are powerful risk factors that predict the onset of SAD symptoms (Clauss and Blackford, 2012; Mulkens and Bögels, 1999; Ollendick and Hirshfeld-Becker, 2002; Öst and Hugdahl, 1981; Ost, 1985; Stemberger et al., 1995). In the first part of my results (discussed above), I proved that an adapted SFC protocol induced short-term social fear memory in NAB rats. This suggests that conditioning events play a role in the development of social fear against conspecifics. However, this fear response is far from being considered pathologic, for instance, is not consolidated for a longer time (rats did not show fear in a robust manner after 24 h) neither showed impaired extinction. Therefore, I tested if combine the SFC (conditioning event) with a second risk factor for SAD promoted a more robust social fear in the rats. I selected the BI, in which the individuals showed an increased sensibility to stimuli (physiological signs of arousal at rest, including higher cortisol levels), and avoid unfamiliar situations (Spence and Rapee, 2016). In this regard, the HAB rats resemble these characteristics. Additionally, I included the LAB rats (opposite in these sense), and the NAB rats, as a control. Then I used the SFC and evaluated the social fear after 24 h.

I found a long-term social fear memory in conditioned HAB rats characterized not only for an emotional memory (fear), as well as social recognition component (social discrimination). These results support my initial hypothesis that combines risk factors enhance the vulnerability to social trauma in individuals with a BI-like trait, suggest high construct validity of the model in rats. Other animal models showed similar results, a rhesus monkey model that classified the subjects by an analogous response to the BI, named “anxious temperament” (AT), animals with higher levels of freezing towards a novel situation and elevated Cort response) (Fox and Kalin, 2014). In the same study, authors reported that in a naturalistic setup (free monkeys), monkeys with a high AT showed reduced conspecifics approaches and maintain long distance between individuals compared to low AT conspecifics (Fox and Kalin, 2014).

Surprisingly, LAB rats also showed long-term social fear memory, which seems to not fit with the originally BI hypothesis. However, considering the abnormal social behavior that usually characterizes the LAB rats, the observed fear could be an additive effect on the already altered sociality of these rats. For named a few examples, LAB rats spend less time in social contact with cage mates (Ohl et al., 2001), showed a lack of social preference after social defeat, and displayed abnormal aggression (see a review in (Neumann et al., 2010)). Moreover, LAB rats showed a deficient performance in social discrimination (Landgraf and Wigger, 2002; Ohl et al., 2002). The SFC

was not the exception, neither the strong pairing induced social discrimination in LAB rats. In this regard, the lack of social discrimination may partially explain their deficit in social interactions, as well (high aggression, few social exploration). Conspecific interactions are indeed shaped by the ability to recognized friends from enemies. In contrast, NAB rats seem to be resilient (no fear neither social discrimination after 24 h). The putative reasons behind it were discussed in above. However, in this context, it is worthy to mention, that NAB rats represent an important tool in terms to identify factors for resilience after social trauma, especial if with are able to directly contrast with populations of vulnerability, such as HAB and LAB rats. In addition, a correlation analysis did not show any significant relation between the EPM variables and the SFC behaviors, which suggest that other factor besides the anxiety are related with the social fear consolidation.

Finally, to clarify if there was any deficit in conditioning learning in NAB rats, or differential pain sensitivity between the rat lines (conditioned NAB rats need more electric foot shocks than HAB rats, it is due to a less sensibility?), I tested the animals in the CFC and HT, respectively. In the CFC, LAB, and NAB rats, either SFC^+ or SFC^- learned and extinguish cued fear normally. In contrast, HAB rats showed an impaired extinction, but this has been already reported (Slattery et al., 2015). Notably, the pre-experience in the SFC increases the sensitivity of the animals, i.e., SFC^+ groups showed higher freezing than the SFC^- groups. In the HT, no significant differences were found between lines. These suggest that the CS-US pairing are independent of the pain sensitivity, and thus, may reflect differences in motivational aspects (see section 4.1.1). In contrast, Jochum and colleagues (2007) described an increase in thermal pain thresholds in HAB rats as compared to LAB and Wistar-control rats in the HT (Jochum et al., 2007). The pre-stress of SFC could induce a ceiling effect in the sensitivity to pain, thus, no differences were observed.

4.3 **Role of the AVP in the consolidation of social fear memory**

It was previously shown, that HAB rats carried an SNP in the AVP gene promoter causing an *in vivo* overexpression of AVP (Murgatroyd et al., 2004). As well, previous findings showed that AVP acts an enhancer of the social recognition (Albers, 2015; Dantzer et al., 1988; Le Moal et al., 1987). Most of these effects seems to be modulated by the V1a receptor (Dantzer et al., 1987; Engelmann, 2008; Engelmann et al., 1994; Landgraf et al., 1995). Besides memory retention, AVP receptor act to discriminate social context, for instance, in the MEA a higher V1a and V1b mRNA expression is induced after being exposed to sick conspecific odor, while expression of OXT receptor mRNA was increased when rats were exposed to healthy conspecific odor (Arakawa et al., 2010).This suggests

a mechanism that mediates the long-term social fear discrimination of conspecifics in SFC. Therefore, to test the hypothesis that AVP contributes the individual recognition and eventually the social fear after SFC, I infused animals either centrally (icv) or locally (lateral septum) synthetic AVP or its receptor antagonist (V1a-A) immediately after the social fear acquisition and evaluated the effects 24 h after on the social fear discrimination test.

In central manipulations of the AVP system, I found that HAB and LAB rats represent the extreme ends of an inverted U-shaped curve in terms of their response to AVP in the consolidation of social-fear memory, *i.e*., HAB/V1aR-A rats and LAB/AVP rats, both reduce social fear consolidation. This mediation it may be due to downstream pathways, since the V1a receptor binding did not differ between lines (Wigger et al., 2004). However, it is difficult to explain the mechanism behind such opposite response between HAB and LAB rats, since icv may affected different brain regions at the same time, and the observed behavior could be the combination output of that network.

Septal manipulations of the AVP system were study since this region mediated the enhanced social recognition effects of AVP. Additionally, the LS seems to control the hierarchical processing between stimuli mediated by AVP. For instance, an increase AVP release enhanced cue processing related with classical conditioning, while impaired the contextual memories (Desmedt et al., 1999). However, LS infusions of V1aR-A in HAB or AVP in LAB rats, did not reveal significant effects. Moreover, HAB rats lost their social discrimination ability. The lack of effects observed can be due to time-dependent differences in the ability of AVP to exert their maximum effects or to a weaker involvement of LS in memory consolidation. In this regard, LS has been involved more in social fear extinction than acquisition (Menon et al., 2018; Zoicas et al., 2014). The previous results showed that AVP contributes only partially to the social fear consolidation, suggesting that both lines can share alternative mechanisms; however, it does not explain NAB rats' resilience.

4.4 **Role of the GC in the SFC: pre- and post-conditioning effects**

My previous findings indicated that AVP contribute only partially to the consolidation of the social fear memory (*i.e*., a bimodal response for HAB and LAB rats, that in none of the cases fully rescued the social investigation or ameliorate the freezing response). Moreover, both HAB and LAB showed vulnerability to acquire for long-term a social fear memory, but the SNP that affects the AVP expression is only carried by HAB rats. This rising the questions of which mechanisms explain the long-term memory both lines? This led me to evaluate the stress-enhanced fear learning hypothesis.

It states that in learning situations, a higher Cort release facilitates the memory consolidation. In simple words, we remember better meaningful events accompanied by emotional response (either positive or negative). In this regard, both lines HAB and LAB showed a dysfunctional HPA axis response, (see a review in (Neumann et al., 2010)). Moreover, Cort administration enhanced the memory of the defeater from one day up to one week in rats after social defeat (Timmer and Sandi, 2010; Weger et al., 2018)

In the first experiment, HAB rats showed higher Cort levels compared to NAB rats in the basal levels. These has not been reported before yet, normally differences were observed after stressors. Surprisingly, the rise of Cort during social fear acquisition was similar between SFC^+ and SFC^- groups, and no line differences were observed. This indicates that the transport and the novelty stimuli (*e.g.*, empty cage, social stimuli) are enough to promote to increases Cort levels in SFC^- rats. Notably, during social fear discrimination only the SFC^+ groups showed a significant rise of Cort levels after the Known stimulus presentation.

In the second experiment, I aimed to study the suppression of adrenocortical activity effects on the long-term social fear consolidation in HAB and LAB rats. Based on the previous results, I hypothesized that the higher basal levels of Cort in HAB rats compared to NAB rats, prime the acquisition of social fear, and promote a long-term social fear memory. Although, due to technical difficulties with the breeding it was not possible assess the Cort levels in the LAB rats at the same time. However, based on previous findings, *i.e*. LAB rats showed higher Cort after social defeat, a similar output is likely in the SFC paradigm. The SFC resembles an adverse social encounter and promote as well social avoidance (Masis-Calvo et al., 2018). Therefore, my prediction was that blocking the levels of Cort before social fear acquisition impairs the long-term social fear memory consolidation in HAB and LAB rats. To reduce the Cort availability I used a treatment with metyrapone (Met), a selective inhibition of CYP11B1 and CYP11B2 activity, that reduces the Cort and aldosterone levels in both rats and humans (Fleseriu and Castinetti, 2016; Rigel et al., 2010).

I found similar social fear acquisition between and within lines comparing Veh *vs* Met groups. This is an important observation since a reduction of the Cort levels do not affect the social approach during the acquisition, suggesting that eventual memory impairs are more related with a consolidation process than with poorly learning (*i.e*., shocks can be taken as the number of approach to a conspecific, that may reflect equal social motivation).

Regarding the social fear discrimination, my predictions were confirmed partially, indeed reduced Cort before social fear acquisition affects the social fear consolidation reflected by the

unaffected levels of social investigation in the SFC^{+}/Met group. I indicated partly, because we could see some levels of freezing, although did not reach a significance and could be dismissed it. Moreover, the data in LAB rats seems to support the hypothesis, since SFC^{+}/Met showed higher social investigation and less freezing than SFC^{+}/Veh. However, is a limited evidence that need to be contrast with appropriate controls (*i.e.*, unconditioned group). These data are in line, with studies that applied metyrapone before training and it reduced the memory fear in pavlovian fear conditioning (Barrett and Gonzalez-Lima, 2004; Cordero et al., 2002; Roozendaall et al., 1996).

The social discrimination results in this experiment are persuasive examples of the effects of "shifting" sides along a gradient of stress response and its impact on social recognition (Fig.31). In other words, follow along the proposed curve of the Yerkes-Dodson law (Lupien et al., 2007). Assuming, the HAB/SFC^{-} group, which so far never showed significant discrimination are imaginary placed in the left side of curve (*i.e.*, putative low levels of stress compared to the SFC^{+} groups). However, after a small stressor (*i.e.*, the ip injection of saline solution), now they show significant recognition because they move forward in the curve and reach an optimal stress level. Interestingly, the other SFC^{-} group failed to replicate this effect due to the metyrapone treatment which is dampening the stress response.

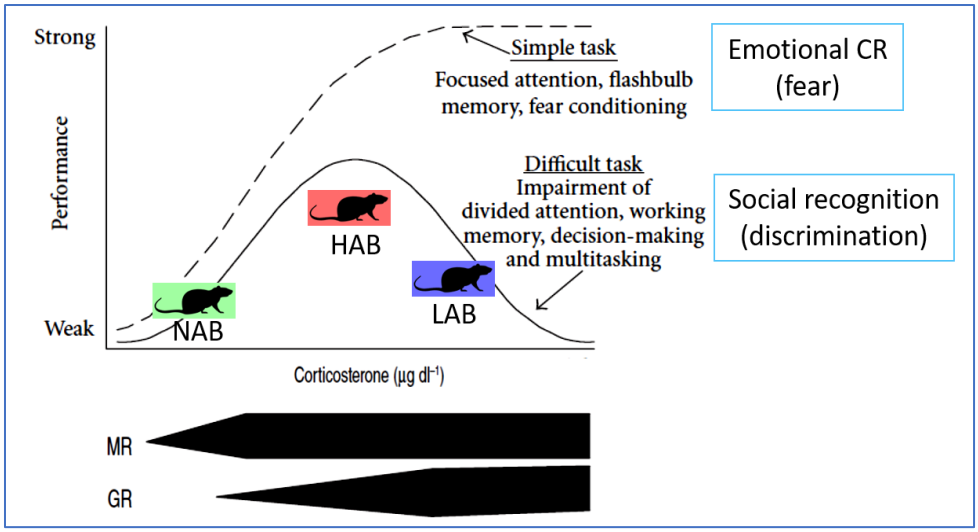


Fig.31. Yerkes-Dobson law: Stress (GC) effects on the social fear discrimination.
Adapted from (Diamond et al., 2007).

In contrast to my previous findings, the SFC^{+}/Veh HAB rats did not show significant discrimination. A similar rationale can be used to explain this discrepancy in the SFC^{+} groups. I speculate that increased stress in SFC^{+}/Veh due to the additive effects of stressors (*i.e.* being conditioned and

receiving an injection), pushed them away from the optimal stress levels of memory performance. The other way around, the SFC^+/ Met neither discriminate because the treatment pulled it down from the optimal levels. Although, I did not measure directly the Cort response to ip injections, several reports in rats agreed on consider considered it a valid stressor with impacts on different memory processes (Kart-Teke et al., 2006; Nagel and Huston, 1988).

Finally, in the third series of experiments, a subtle GCs effects were observed on the consolidation of NAB rats. Although, no treatment effects but the previous stress due to experimental procedures (*e.g*., handling, and ip injection) induced long-term social fear in NAB rats. This point out to the "pre-exposure stress" effect that enhance fear consolidation (Fanselow and Ponnusamy, 2008). For instance, pre-exposure to stress in rats (*i.e.* 15 electric foot shocks) enhanced the context fear conditioning, even using a single CS-US pair (Rau et al., 2005). This sensitization seems independent of the stressor kind, for instance, forced swim stress enhances the acquisition of the conditional eyeblink response, as well, restrain stress enhances the response to context fear conditioning (Fanselow and Ponnusamy, 2008). Unfortunately, the experimental pilots in NAB rats were based on a very limited size sample; hence, my conclusions are equally limited. Future studies are needed it to select a better handling and increase the sample size. Another option would be stressing the animals straightforward and evaluated the endogenous release of Cort in the SFC.

4.5 Brain activation (cFos) during social fear discrimination in NAB and HAB rats

In this study we aimed to identify the brain subregions of the hippocampus and amygdala involved in the social fear discrimination between HAB and NAB rats. For that purpose, I selected the cFos immunohistochemistry, since this Immediate-early gene are helpful to identify the activation of brain areas related to a specific behavior, such as fear or stress responses (Martinez et al., 2002). Within the hippocampus, I only found a significant increase of c-Fos positive cells in the CA2/3 regions in NAB/SFC^- group compared to NAB/SFC^+ group, measured 6 h after social fear acquisition. This activation explained a 39% of the variance observed in the social investigation between this two groups. Regarding the line effects, I did not detect differences between HAB vs NAB in any brain area. Similarly, social defeat studies did not found differences between HAB and LAB rats in any subregion of the dorsal hippocampus (Frank et al., 2006). This experiment was restricted to the dorsal part of the hippocampus. However, the ventral part has been associated with anxiety-related behaviors (Bannerman et al., 2004; Bertoglio et al., 2006). This region potentially reveals differences between HAB and NAB rats, since the major phenotype feature that distinct them is precisely the

trait of anxiety-related behavior. Finally, it would be important to address the limitations of this study since we only use a c-Fos marker we could not distinguish between neurons and glia cells. Therefore, a double staining (e.g. use of antibodies that serve as markers of either neuronal or glia cell bodies) to label a specific cell type is necessary (Tian and Bishop, 2002; Bennett and Schwartz, 1994). This missing separation could lead to our observation of no differences between groups (SFC^+ vs SFC^-; HAB vs NAB). Second, a proper control of the social activation is missing, meaning a animal that was not expose to social stimuli could help to reveal relevant differences between groups.

4.6 **Concluding remarks and future perspectives in SFC in rats**

4.6.1 Individual differences in SFC: a promise model to identify susceptible versus resilience subpopulations to social trauma

Individual differences have always captured our attention, considering that only 20% of the persons exposed to traumatic events develop psychiatric disorders (Fanselow and Ponnusamy, 2008). Here we could see how differential anxiety-related traits (HAB and LAB rats) showed higher vulnerability compared to NAB rats. Even more we could identify some inherent factors to each rat line (e.g. AVP or GC levels), that may contribute to the social fear consolidation, and then evaluated them in the NAB rats to promote a pathological response. These offers a powerful approach to identify other genetic, or environmental factors relevant for the etiology and treatment of SAD.

4.6.2 Network involve in the SFC model

SFC is a relatively novel model (Toth et al., 2012b) compared to the traditional conditioning protocols. Currently, the information regarding the brain regions engaged in the different SFC phases (acquisition, consolidation, and recall) is limited to cFos studies in female mice in a few selected areas, such as the amygdala, lateral septum and somatosensorial cortex (Menon et al., 2018). Currently, efforts either in male mice (Grossmann et al., in preparation) as well in rats are quantifying the cFos expression in a few more selected areas (Masís-Calvo et al., in preparation). However, I still need to include promise targets such as the BNST, a center for the integration of information originating from the amygdala, and the hippocampus (Steimer, 2002). Indeed, SAD patients showed an increase in phasic activation of this region trigger by the expectation of aversive events compared to healthy controls (Figel et al., 2019). After the strong response observed in the freezing time in rats after the SFC, another promising target would be the PAG, due to its intrinsic control of this response. Nevertheless, provide a functional network become a more challenging

task than only identify other regions. For instance, separate the contribution of the Pavlovian vs the passive avoidance conditioning is really difficult (LeDoux et al., 2017). Further, in the SFC we are dealing with a complex stimulus, as a conspecific, which may require not only a multimodal encoding process but itself is a source of variability (*e.g.* stimuli rats may emit UV´s that given either positive or negative feedback to experimental subject).

Part II: Contribution of NPS to cued fear extinction and the stress response in virgin and lactating females

Sex differences are an important factor for the etiology of anxiety, trauma, and stressor-related mental disorders, such as PTSD and SAD, in which woman are twice likely to be affected compared to men (Blume et al., 2017; Shansky, 2015; Stockhorst and Antov, 2016). However, there is still a strong bias towards male preclinical research (Milad and Quirk, 2012). This depicts a lack of understanding of the contributing mechanisms that are specific to the female brain, which in turn delays the possibility of treatment improvement for women´s healthcare (Galea et al., 2020; Shansky, 2015). The NPS system is not an exception, it was first described in 2002 (Sato et al., 2002) and ten years have elapsed until the emotional regulation of this neuropeptide was studied in females (Wegener et al., 2012). Furthermore, none of the existing studies consider the contribution of the female reproductive states experienced throughout life, such as natural cycling and the period of lactation (Germer et al., 2019; Kreutzmann et al., 2020). Therefore, in the second part of my thesis, I aimed to characterize the central NPS-system in cycling virgin and lactating female rats. In detail, I describe the basal NPS/NPSR expression (on the level of mRNA) in stress-related brain regions, the effects of NPS in extinction of cued fear, as well as its effects on peripheral stress biomarkers (Cort, OXT, and NPS).

4.7 *NPS* expression is upregulated in stress-related areas in lactating compared to virgin females

As mentioned above, the general aim in this second project was to understand how the NPS system is regulated in different reproductive states of the female brain. In a first approach, I compared the basal *mRNA* expression of *NPS* and its receptor in stress-related areas of virgin and lactating females. I found an upregulated *NPS* expression in the LC, PVN, and AMY in lactating compared to virgin females, while the *NPSR* expression remained unaltered. Previous studies in males describe *NPS* expression in the LC and AMY (Xu et al., 2007; Xu et al., 2004). Although Xu and colleagues did not detected *NPS* expression in the PVN, it was observed in the dorsomedial hypothalamic nucleus (DMH) (Xu et al., 2004), which keep intra-hypothalamic projections to the PVN (Ter Horst and Luiten, 1987). Retrograde tracer studies in our laboratory, also identified NPS afferents from the LC to the PVN of male rats (Grund et al., 2017). Thus, the observed expression of *NPS* in the PVN of female rats can be explained by axonal transport either from the DMH or from brainstem afferents that

have been described in both mice and rats (Clark et al., 2011; Grund et al., 2017). The axonal transport consists of different mechanisms that move proteins, organelles, or *mRNAs* from the neuronal soma to distal axonal compartments. Axonal transport helps the neurons to overcome longer distances by localizing mRNAs to synapses and locally producing proteins, as well this strategy provide a tight and rapid regulation of synaptic protein abundance in space and time (reviewed in (Biever et al., 2019; Hurtley, 2019)). Recently these mechanisms gained more attention due to their implications in neurodegenerative diseases, such as alzheimer's disease, amyotrophic lateral sclerosis, and inclusion body myopathy (Alami et al., 2014; De Vos et al., 2008). Other important stress-related modulators, such as AVP, OXT, and Brain-derived neurotrophic factor also show axonal transport of the mRNAs (Conner et al., 1997; Mohr et al., 1991).

In contrast, both virgin and lactating females showed comparable expression of *NPSR* in the LC*,* although, studies in male rats did not reveal detectable mRNA levels in the LC (Xu et al., 2007; Xu et al., 2004). This finding hints towards a potential sex difference. However, one needs to consider that differences in expression patterns might be due to the alternative technique approaches used: In the present thesis, female mRNA levels were analyzed using qPCR, whereas mRNA in male rats was assessed via in situ hybridization. Similarly to males, *NPSR* expression was found in the PVN and AMY (Xu et al., 2007), however, there was no significant difference when comparing females of different reproductive states. Within the PVN, the NPS system seems to interact with the OXT system (Grund et al 2017). OXT is well known not only as a maternal neuropeptide, but also as stress modulator (Jurek and Neumann, 2018; Jurek et al., 2012; Slattery and Neumann, 2008). Grund and colleagues showed that *NPSR* mRNA is mainly expressed in OXTergic neurons of the PVN (Grund et al., 2017). Moreover, a calcium imaging *in vitro* assay showed that OXT neurons are activated after NPS stimulation. In the present thesis, I also found that central NPS administration promotes OXT release into the periphery (blood plasma; discussed in section 4.9), suggesting that part of the peripheral OXT derives either from the PVN or SON (Jurek and Neumann, 2018).

Furthermore, NPS neurons from LC also project to the AMY (Clark et al., 2011), hich is one of the regions with high NPSR expression (Cohen et al., 2018; Xu et al., 2007). NPSR activation in this region promotes anxiolytic effects in male mice (Jüngling et al., 2008). The anxiolysis is mediated by an enhanced glutamatergic transmission into GABAergic neurons of the intercalated cell mass, ultimately resulting in inhibition of the CEA outputs (Jüngling et al., 2008). Similarly, the mechanism by which OXT promotes anxiolysis and reduces fear, OXTergic PVN projections to the CEA also

enhance GABAergic signaling that silence the CEA outputs (Knobloch et al., 2012; Rickenbacher et al., 2017).

To date, this is the first study revealing differential *NPS* expression in lactating rats. Lactation is a period of dramatic brain plasticity, however, its underlying genetic basis is still poorly understood (Gammie et al., 2016; Kuroda et al., 2011). The NPS upregulation in lactating females suggests a contribution of this neuropeptide system to maternal adaptations, such as stress modulation (Neumann et al., 1998a; Slattery and Neumann, 2008; Stern et al., 1973). This hypothesis is supported not only by the consistent upregulation present in all examined stress-related areas, but also because of the fact that NPS is involved in other aspects of the stress response: *i.e*., NPS neurons are activated after stressor exposure (c-Fos)(Adori et al., 2016; Ebner et al., 2011), NPS infusions are promoting GC release (Alami et al., 2014; Smith et al., 2014), and stress-induced analgesia cascades (see review in (Lee et al., 2020). Furthermore, CRF stress-induced release activates NPS neurons in the LC (via CRF receptor 1), which in turn activates the NPS release in downstream areas, such as PVN and AMY (Jüngling et al., 2012).

4.8 Facilitation of cued fear extinction by NPS depends on the female reproductive state

To evaluate the putative contribution of the observed *NPS* upregulation to the behavioral response, I selected the CFC paradigm to compare the fear response between lactating and virgin females. Previously in our laboratory, lactating mice showed a reduced cued fear response in acquisition and extinction compared to virgin mice. This effect was shown to be mediated by the high activation of the endogenous OXT system during lactation (Menon et al., 2018). Moreover, previous reports showed that central NPS administration in male mice and rats facilitates cue fear extinction (Jüngling et al., 2008; Slattery et al., 2015). Based on these findings, I hypothesize a similar behavioral response in lactating compared to virgin females (reduced fear), that may be abolish by icv NPSR-A treatment. Whereas in virgin females (putative more fearful than mothers), I expect a similar facilitation of fear extinction after icv NPS treatment, as described in male rats (Slattery et al., 2015). In addition, I grouped the virgin females according to their estrus cycle in **high** (**HE**; proestrus and estrus) and **low** (**LE**; metaestrus and diestrus) estradiol groups, since compelling literature indicates that estradiol plays a key role in modulation of fear extinction (Milad et al., 2009; Stockhorst and Antov, 2016).

Cued fear acquisition

No effects of the reproductive state (lactating *vs* LE or HE virgin females) were found during acquisition of cued fear in female rats. This is in contrast with the CFC data in female mice (Menon et al., 2018), suggesting a species-specific regulation in mice and rats. Although, the expected reduced fear in lactating rats, which is suggested to be due to the highly activated OXT system, is in contrast to male rats evidence, where central application of OXT prior to acquisition does not affect the CR (*i.e*., similar freezing response in OXT and Veh-treated groups)(Toth et al., 2012a).

Cued fear extinction

During extinction of cued fear, freezing levels in all females decreased over the subsequent CS presentations, depicting a successful extinction of cued fear. Here, lactating females and LE virgins showed a general trend for a reduction in the freezing response over CS presentations, whereas the HE virgins already showed a low freezing response at the first CS presentation. Studies in rodents and naturally cycling women suggest that fluctuations of the menstrual cycle hormones alter the fear extinction (Milad and Quirk, 2012). Here, estradiol plays an important role, since exogenous estradiol administration facilitates extinction of cued fear in rats (Milad et al., 2009).

Regarding the reproductive state, group contrast (Lactating vs LE vs HE) showed a strong trend. Main effects analysis showed that HE virgins differ from the other groups (HE rats showed low freezing and no response to activation or blockage of the NPSR), while lactating females behave in a similar way to the LE virgin females (reduced freezing after NPS). This can be explained by the similar hormonal profile between LE virgins and lactating females. In lactating females, estrogen and progesterone levels drop after parturition and remain low until postnatal day five (Hansen et al., 1983; Stolzenberg et al., 2019). Only few studies are comparing lactating *vs* virgin females in classical conditioning and their reports are ambiguous. On one hand, Rima and colleagues found a reduced fear in lactating females (without pup presence) compared to virgin females, in Sprague Dawley (Rima et al., 2009). On the other hand, a study in Long Evans rats reports that a reduced fear responses in lactating females require the presence of the pups, since lactating females in absence of their pups behave in the same manner as virgin females (Rickenbacher et al., 2017).

The latter study is in line with my findings in Wistar rats, the fear response was measured in absence of the dam´s pups. Moreover, I used similar conditioning settings to those employed by Rickenbacker and colleagues (*i.e*., 3 CS-US pairings, foot shock intensity and extinction context with low adverse conditions). In contrast, Rima and colleagues, applied a strong adverse conditioning in

their protocol (*i.e.*, 13 CS-US pairings, the US was a predator call). Furthermore, most of the studies that report reduced fear responses in mothers, using the acoustic startle response, were conducted in the presence of pups (Ferreira et al., 2002; Hård and Hansen, 1985). A reduced fear in the appropriate context (pups presence) improve several maternal skills relevant for the fitness of the mothers, such as hunting and foraging, social awareness (pup cues and conspecifics valence), and aggression performance (Fleming and Luebke, 1981; Rickenbacher et al., 2017; Rima et al., 2009). Future studies should evaluate the dynamic release of NPS in lactating rats within the PVN in the CFC context with or without pups being present. Finally, strain differences cannot be ruled out in this sense, since all three experiments were carried out in different strains.

Main effects of treatment analysis (Fig. 27 F) showed that NPS significantly reduced the freezing response in lactating females, while in LE virgin I found only a biological trend to freezing reduction. These central effects are most likely driven by AMY activation, as previous studies localized this NPS-mediated effects in the BLA (Jüngling et al., 2008; Meis et al., 2008). Unexpectedly, NPSR-A treatment did not alter freezing in lactating females, while in LE virgin females it increased the freezing levels compared to both, NPS (significant) and Veh (strong trend) treatment One can speculate that this is due to higher NPS availability as product of the upregulation mentioned above (yet to be confirmed by protein level analysis). In contrast, HE virgin females did not show differences between treatments.

The latter results highlight the role of the estradiol levels in the cued fear extinction, these results are in line with previous findings in female rats, where high estradiol levels promoted faster extinction of cued fear (Milad et al., 2009; Trask et al., 2020), whereas low estradiol levels reduced it (Rey et al., 2014; Trask et al., 2020). Moreover, estrogen agonists facilitate consolidation of extinction of cued fear (Zeidan et al., 2011). One mechanism behind, seem to be a shifting between the inhibitory– excitatory balance in subregions of the AMY. Blume and colleagues describe a high inhibition of the lateral AMY that facilitates the extinction of cued fear during high estradiol states (Blume et al., 2017). The different process by which estradiol promotes such inhibition are not fully understood yet, and the interactions of the estradiol with other stress system is complex (for a review see (Stockhorst and Antov, 2016)).

Additionally, it was noteworthy, that in general, the freezing response in females was lower than in males, which were conditioned using the same protocol (Slattery et al., 2015; Toth et al., 2012a). Recently, this fact is being criticized as females may display more active coping styles than males (Gruene et al., 2015). This argument is in line with my personal observation during

experiments, as most of the tested females actively tried to escape the conditioning chamber by jumping onto the walls during CS presentation instead of freezing.

Taken together, manipulations of the NPS system may be useful to treat deficits in fear extinction learning in females exhibiting a low estradiol profile. The reduced freezing response in the HE virgin females, and the apparently missing treatment response, remarks the high ability of extinguish fear during this period. An extreme example of this is the ameliorate symptom in Schizophrenic patient during high-estradiol phases (Bergemann et al., 2007). This hormonal variation should be taken into consideration for therapeutic strategies in preclinical studies and especially women.

4.9 **Effects of NPS on the physiological stress response: Cort and OXT levels**

Central NPSR activation or its blockade was evaluated in female virgin and lactating (PND 9-10) rats. 20 min post-infusion, rats were sacrificed to collected trunk blood samples that were analyzed for plasma Cort, OXT, and NPS concentrations. I found that central NPS treatment increased peripheral Cort levels in virgin females but failed to induce Cort changes in lactating females. Furthermore, the data gathered in a pilot study that monitoring plasma Cort release by JVC sampling, confirmed that NPS treatment in virgin females significant increased Cort levels compared to the Veh group, while lactating females did not show response to the NPSR-A treatment. The response in virgins is in line with observations in male rats measured at a similar time point after NPS administration (Smith et al., 2006). Considering the stress hypo-responsivity during lactation, the missing effect of NPS in lactating females was expected as a typical hallmark of this period (Brunton and Russell, 2008; Neumann, 2001; Neumann et al., 2001; Slattery and Neumann, 2008).

Moreover, RIA analysis of a subset of the plasma samples revealed that either virgin as well as lactating females showed a similar increase in plasma OXT levels after NPS treatment. In general, plasma OXT levels indicate the activation of the magnocellular OXTergic neurons within PVN and SON, from which the nonapeptide is transport through neurohypophysis into the periphery. Several stimuli promote peripheral OXT release, such birth, suckling, stressors exposure and hyperosmotic stimulation, for named few (see an extend review in (Jurek and Neumann, 2018)). Since females were measure under basal conditions (no evident stress) and mothers were separated from their pups (no suckling stimulation), the increased OXT must be mainly induced by the NPS action. As I mentioned, OXTergic neurons in the PVN express NPSRs, and I already showed that virgin and lactating females did not differ in the relative expression of the receptor in this region, which may

explain the similar response in both groups. Finally, this is the first time that NPS protein was quantified by ELISA, although no significant differences were observed, establishing for a proper method to measure plasma NPS content will be useful for future projects. So far, there is no information about the expected values of this icosapeptide in the plasma of rats available. However, NPSR is expressed in several peripheral tissues (see a review in (Zhang and Tao, 2019), and in rats one of the highest abundance is found in mammary and salivary glands (Xu et al., 2004), but its functional aspects have not been deciphered yet.

4.10 **Concluding remarks and future perspectives: NPS female studies**

In summary, *NPS* expression was shown to be differentially regulated between lactating and virgin females in stress-related regions. Moreover, in LE virgin and lactating females, NPS treatment facilitated fear extinction, while its blockage delayed it. In contrast, HE females showed the lowest freezing levels compared to the other groups, suggesting a trauma-protective effect of estradiol. This suggests that anxiolytic effects of NPS are sensitive to variations in sex hormones. However, the exact mechanism by which the estradiol and NPS systems interact still needs to be elucidated. Furthermore, I showed that peripheral stress parameters, such as Cort and OXT, positively correlate with central NPS administration in virgin females, while in lactating females only OXT levels are increased. Altogether, these findings highlight the relevance of the neuropeptide NPS as a potential treatment for stress-related disorders and as a maternal modulator. In this regard, NPS combines a series of advantages from a translational point of view, *i.e.*, it can reach the brain by intranasal applications (reference), some NPS antagonists can even pass the blood-brain barrier (reference), and its promnesic and anxiolytic effects are not accompanied by sedative side effects, such as the traditional benzodiazepines (Dine et al., 2015; Ionescu et al., 2012; Melamed et al., 2010; Sartori et al., 2016; Singewald and Holmes, 2019).

As mentioned above, I quantified relative mRNA levels of NPS and NPSR within various stress-relevant brain regions. However, due to the lack of specific antibodies available, protein quantification and thereby confirmation of the observed alterations was not executed so far. Currently, I focus on measuring NPS and NPSR protein levels in the same brain regions examined above. To my knowledge, no study examined central NPS release in response lactating and virgin females yet. Hence, I aim to quantify NPS protein levels in the cerebrospinal fluid of virgin and lactating females under basal and stressed conditions by ELISA. Moreover, future studies are needed to identify the putative function induced by the seen upregulation of *NPS* expression in lactating

females. A plausible behavioral aspect, which might be mediated by central NPS activity, is maternal behavior. Hence, observation of maternal behavior following pharmacological manipulations of the NPS system is an essential readout parameter. This is underpinned by the fact that some of the behavioral adaptations during lactation might overlap with behaviors influenced by NPS (see a review in (Grund and Neumann, 2019; Zhang and Tao, 2019). For instance, maternal aggression (Bosch and Neumann, 2012; Erskine et al., 1978; Klampfl et al., 2013), reduced anxiety-like behaviors (Ferreira et al., 1989; Jurek et al., 2012; Neumann et al., 1999; Pereira et al., 2005), adjusted food consumption(Leon and Woodside, 1983) and altered sleep-cycles (Benedetto et al., 2017) would be interesting behavioral facets to study.

5. References

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MaMa

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2017 International summer school: "Stress and Cognition: From basic mechanisms to psychopathology". Radboud University. Nijmegen, The Netherlands.
2016 FELASA training, category B, Regensburg University. Bavaria, Germany.
2014 IV Regional Scientific Association meeting: ACCMAL / FESSACAL / FESAHANCCCAL / ICLAS. Oral presentation. San Jose, Costa Rica.
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Appendix I

Solution	Composition	Company
4% PFA in 1x PBS. pH 7.4	40 g Paraformaldehyde	Merck
	Fill up to 1 L with 1x PBS	
Cryo protecting solution 500 ml	150 ml Ethylenglycol	Acros organics
	150 ml Glycerol	Fisher Scientific UK
	Fill up to 500 ml PBS 1x	
0.1 M PBS pH 7.4 (10x solution)	80 g NaCl	Fisher Scientific UK
	2 g KCL	Merck
	14.4 g Na_2HPO_4	Merck
	2.4 g KH_2PO_4	Merck
	800 ml ddH_2O	
	adjust pH to 7.4. add ddH_2O to final Volume of 1L	
H_2O_2 solution	3% H_2O_2	Sigma-Aldrich. concentration 35%
	10% Methanol	AnalaR NORMAPUR. VWR Chemicals. Radnor. Pennsylvania. USA
	Fill up with PBS 1x	
Blocking solution 1	0.5% Triton-X-100	Sigma-Aldrich
	5% Normal Goat Serum	S-1000. Vector Laboratories. Burlingame. CA. USA
	Fill up with PBS 1x	
Blocking solution 2	0.5% Triton-X-100	Sigma-Aldrich
	2% Normal Goat Serum	S-1000. Vector Laboratories. Burlingame. CA. USA
	Fill up with PBS 1x	
avidin-biotin solution Vectastain ABC Kit. Peroxidase (HRP)	90 µl Reagent A (Avidin)	PK-4000. Vector Laboratories. Burlingame. CA. USA
	90 µl Reagent B (Biotinylated HRP)	
	Fill up to 20 ml with PBS 1x	
DAB Substrate Kit Peroxidase (HRP) with Nickel	5 ml ddH_2O	SK-41000. Vector Laboratories. Burlingame. CA. USA
	84 µl Buffer stock solution	
	100 µl DAB stock solution	
	80 µl H_2O_2 solution	
	80µl Nickel solution	
Roti®-Histokitt		Carl Roth GmbH & Co. KG

Appendix II

Oxytocin did not reduce the social fear in SFC⁺ HAB rats

Oxytocin proved to has remarkable rescue effects in the social investigation levels of conditioned (SFC⁺) mice infused either centrally or in the Lateral septum (Toth *et al.,* 2012, Menon *et al*., 2018). In rats, central oxytocin also rescued the social avoidance in socially defeated subjects (Lukas *et al*., 2011). Therefore, I tested if an icv infusion of OXT (50 ng/5µl; 10 min prior extinction) influences the social fear in rats after SFC. No differences were found in CS-US pairings between the SFC⁺/Veh and SFC⁺/OXT group (Fig. S1. A, *U*= 54; *p*= 0.346). During social fear discrimination, I found significant differences between groups in the social investigation and freezing time (Table S1). *Post-hoc* analysis revealed a significant reduction of the social investigation in both conditioned groups (SFC⁺/Veh and SFC⁺/OXT) compared to SFC⁻/Veh (Fig. S1.B), but not treatment effects between SFC⁺ groups (Table S1). In contrats, conditioned groups showed singnificantly higher freezing time compared to SFC⁻/Veh group, however, no treatment effects were observed between SFC⁺ groups (Table S1). Furthermore, OXT treatment impaired the social discrimination in SFC⁺ rats (Fig. S1.D, *p*=0.812), since SFC⁺/Veh groups showed social discrimination (*p*= 0.031). As a disclosure for further discussion, here, the vehicle controls for SFC⁻ and SFC⁺, although, were evaluate the same day as the SFC⁺/OXT group during the social fear discrimination test, they were controls for the AVP experiment (described below), thus, the infusions were done right after social fear acquisition. Therefore, can be used as reference values, however, they did not represent the ideal controls for the OXT group that was infuse before social fear discrimination.

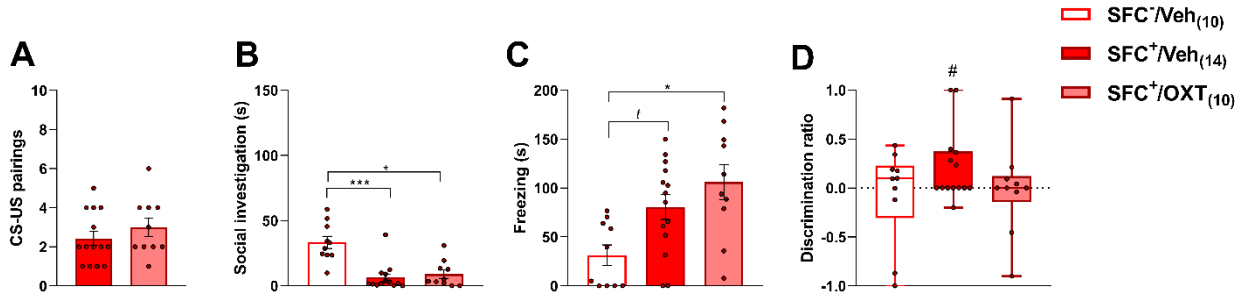

Fig. S1.Experimental pilot to evaluate the Oxytocin (OXT) effects in the social fear discrimination in conditioned HAB rats. A) social fear acquisition (CS-US pairings). B) Social investigation during social fear discrimination. C) Freezing time during social fear discrimination. D) Discrimination ratio. *p<0.05, ***p<0.001 and ᵗp=0.063 indicated comparisons between groups. #p<0.05 indicated significant difference with critical value (zero) in the One sample t-test.

Table S1. Statistics for social fear discrimination trial between HAB rats.

	Social investigation	**Freezing time**
Group comparisons (Kruskal-Wallis test)	H=15.98 p<0.0003***	H=10.34 p<0.005**
Post-hoc (Dunn's test)	p values	p values
SFC^-/Veh vs. SFC^+/Veh	0.0003***	0.063^t
SFC^-/Veh vs. SFC^+/OXT	0.010*	0.005*
SFC^+/Veh vs. SFC^+/OXT	>0.999	0.854